Original paper

Beta2-adrenergic receptor agonist inhibits keratinocyte proliferation by mechanisms involving nitric oxide

Chieh-Shan Wu[1], Der-An Tsao[2], Huoy-Rou Chang[3]

[1]Department of Dermatology, Kaohsiung Veterans General Hospital, Kaohsiung city, Taiwan
[2]Department of Medical Technology, Fooyin University, Kaohsiung city, Taiwan
[3]Department of Biomedical Engineering, College of Medicine, I-Shou University, Kaohsiung city, Taiwan

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Abstract

Introduction: Beta2-adrenoceptors regulate proliferation of keratinocytes. Nitric oxide (NO) produced by keratinocytes through stimulation of nitric oxide synthase (NOS) mediates keratinocyte proliferation.
Aim: In this study, the mechanism interaction β-ARs and NO production on keratinocyte will be explored, and the important for proliferation will be studied.
Material and methods: To understand the relationship among β2-adrenoceptors, NO production and proliferation in keratinocytes, the experiment is divided to two parts. In the first part of the experiment, keratinocytes are divided into five groups which are treated with 0 M, 10^{-7} M, 10^{-6} M, 5×10^{-6} M and 10^{-5} M isoproterenol, respectively. In the second part of the experiment, the keratinocytes are divided into five groups which are treated with 10^{-5} M isoproterenol and L-NMMA at doses of 0 M, 10^{-6} M, 5×10^{-6} M, 10^{-5} M and 5×10^{-5} M, respectively. We examine NOS expression, NO production, c-AMP level and proliferation in human keratinocytes.
Results: The results show that isoproterenol results in iNOS and ncNOS protein raised and the elevation of nitric oxide. L-NMMA can block the increase of iNOS and ncNOS protein expression and the ability to inhibit proliferation caused by isoproterenol.
Conclusions: Beta2-adrenergic receptor agonist mediates nitric oxide synthase to affect keratinocyte proliferation in skin. The physiological and pathological relationship of these discoveries remains to be defined. These results can provide new possibilities in the therapy of integumentary disease conditions linked with the dysfunction of β-AR-mediated NO production.

Key words: β2-adrenoceptors, nitric oxide synthase (NOS), nitric oxide (NO), keratinocyte, proliferation.

Introduction

Beta-adrenergic receptors (β-AR) are presented on a wide range of tissues and are identified as crucial functional regulators in cardiac, vascular, endocrine, pulmonary, and central nervous systems. Of the several identified classes of β-AR (β1, β2, and β3), it is of particular interest to note that human keratinocytes express only β2 adrenergic receptors (β2-AR) [1]. The β2-AR delivers signals via a G-protein coupled signalling concatenation that includes adenylate cyclase-mediated increases in cAMP [2]. The studies suggested the regulation of keratinocyte proliferation by intracellular levels of cAMP, and that a raise in intracellular cAMP by catecholamine stimulation caused a reduction in proliferation [3–5]. The studies also proposed that a reduction in the capability of psoriatic keratinocytes to respond to β adrenergic agonists with a raise in cAMP could be, in part, responsible for the increase in cell proliferative diseases [6, 7].

Nitric oxide (NO) is synthesized from L-arginine, nicotinamide adenine dinucleotide phosphate (NADPH), and molecular oxygen by NO synthase (NOS) [8–12]. Three isoforms of NOS have been suggested in human tissues. Neuronal NOS (ncNOS) and endothelial NOS (ecNOS) are constitutively presented in a wide range of cells, are Ca^{2+}/calmodulin-dependent, and generate NO to regulate vasodilation and neurotransmission. The third form of NOS, inducible NOS (iNOS), a Ca^{2+}/calmodulin-independent enzyme, has been related with regulation of some cytostatic and cytotoxic effects of the immune system [9, 10, 13–15]. NO donors have been used to inhibit proliferation, without producing significant cytotoxic effects,

Address for correspondence: Prof. Huoy-Rou Chang PhD, Department of Biomedical Engineering, I-Shou University, 1 Section, 1 Shiuecheng Road, Dashu Shiang, Kaohsiung City, Taiwan, phone: 886-7-6151100, ext. 7474, fax: 886-7-3419929, e-mail: huoy@isu.edu.tw, Assoc. Prof. Der-An Tsao PhD, Department Medical Technology, Fooyin University, 151 Chinhsueh Rd, Ta-liao, Kaohsiung, Taiwan, phone: 886-7-7811151 ext. 5416, fax: 886-7-3419929, e-mail: sc095@fy.edu.tw
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of a large variety of cell types, including those obtained from varying normal tissues and diverse tumours [16]. In the human epidermis, keratinocytes are able to present iNOS and ncNOS enzymatic activities resulting in the formation of NO [17, 18]. Marletta *et al.* [19] suggested that epidermal keratinocytes (HaCaT cells) with NO treatment were associated with a biphasic effect on cell proliferation, low dosages of NO donors mediated a proliferative signal to the cells, whereas high levels were cytostatic.

There is evidence that cAMP/PKA signalling pathway is involved in β-ARs-mediated NO generation from endothelial cell studies [20]. Some studies proposed that β-ARs and NO were interrelated and that, in many cases, stimulation of β-ARs was directly connected to NO production [21]. Boyce and Ham [22] reported that endothelial β-ARs-cAMP-NO pathway regulated vasodilatation. Some studies proposed that β2-ARs, through activation of the L-arginine/NO system, were able to conduce to regulation of platelet adhesion to the vessel wall [23–25]. At present, the relationship between β-ARs and NO in skin is not clear. In this study, the coupling mechanisms of β-ARs to NO generation in keratinocytes will be explored, and their importance on the proliferation will be investigated.

Material and methods

Cell culture

Keratinocytes were isolated enzymatically from neonatal foreskins in complete keratinocyte growth media (keratinocyte-SFM, keratinocyte basal medium supplemented with 1 × 2.5 µg human recombinant EGF, and 1 × 25 mg bovine pituitary extract; GIBCO, Invitrogen Corporation, Carlsbad, CA) as described previously [26]. Briefly, the epidermal sheet was separated from the dermal part after treatment at 37°C for 25 min with neutral protease (Dispase II; Roche) and cells were cultured until 80% confluence and then subcultured into 6-cm and 9-cm culture dishes for the experiment. Cell viability throughout the experiments was > 95% as judged by Trypan blue exclusion. In the first part of the experiment, isoproterenol doses of 0 M, 10^{-7} M, 10^{-6} M, 5×10^{-6} M and 10^{-5} M were applied. The keratinocytes were divided into five groups which were treated with 0 M, 10^{-7} M, 10^{-6} M, 5×10^{-6} M and 10^{-5} M, respectively. In the second part of the experiment, the keratinocytes were divided into five groups which were treated with 10^{-5} M isoproterenol and L-NMMA at doses of 0 M, 10^{-6} M, 5×10^{-6} M, 10^{-5} M and 5×10^{-5} M, respectively. The cells for Western blotting assay and the medium for the determination of NO were prepared 24 h after experimental exposure.

Protein measurement

Protein was determined according to the method of Frank *et al.* [27] using bovine serum albumin (BSA) as the standard.

Western blotting

Treated keratinocytes were scraped in lysis buffer comprising 20 mM Tris-HCl (pH 7.4), 2 mM EGTA, 5 mM EDTA, 500 µM sodium orthovanadate (Na_3VO_4), 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 10 µg/ml aprotinin (Sigma), 10 µg/ml leupeptin (Sigma) and 1mM PMSF (Sigma) for 20 min on ice. The products of lysis of cells were centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant of lysates was isolated, the level of proteins was determined and the pellet was discarded. The keratinocyte supernatants were diluted with SDS sample buffer (0.125 M Tris-HCl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, pH 6.8) and placed in a boiling water bath for 5 min before electrophoresis. Aliquots corresponding to 50 µg protein were applied to 12% SDS-polyacrylamide gels. Western blotting consisted of overnight electroblotting of the proteins from the polyacrylamide gel onto nitrocellulose filter paper. After blocking with 5% non-fat dried milk (2 h at room temperature), the nitrocellulose sheet was incubated with a 1 : 500 dilution of polyclonal rabbit IgG raised against ncNOS or iNOS and detected with a 1 : 3000 dilution of horseradish peroxidase-conjugated goat antibody to rabbit IgG. For quantification, the images were recorded on a digital imaging system (Alpha Imager 2000, Alpha Innotech Corporation, San Leandro, CA) and analysed densitometrically (Alpha Ease ver. 3.23, Alpha Innotech Corporation).

NO assay

Nitrite levels were determined by the modified Griess reaction to measure total amounts of NO released from the keratinocytes [28]. Cultured keratinocytes were treated with different experimental conditions and incubated for 24 h. The supernatants were collected and centrifuged to remove cell crap. Samples and sodium nitrate standards were loaded at 50 µl/well into microtitre plates and reaction buffer (50 mM morpholinopropanesulfonic acid, 1 mM EDTA, pH 7.0) was added to a final volume of 85 µl.

Then 5 µl nitrate reductase (0.01 U/well; Sigma, St. Louis, MO) and 10 µl 2 mM β-NADH (reduced form, Sigma) were added to each well. The plate was placed on an orbital shaker and incubated at room temperature for 20 min. Subsequently, 50 µl colour reagent A (sulfanilamide dissolved in 3N HCl) and an equal volume of colour reagent B [N-(1-naphthyl)ethylenediamine dihydrochloride dissolved in H_2O] were added to each well and this was followed by incubation at room temperature for 5 min. The optical density was determined with a microtitre plate reader (MRX-II, Dynex Technology, Chantilly, VA) at 540 nm and then the value of the blank controls (medium without cells) was subtracted. The NO levels were estimated from the standard curve.

Total cellular cAMP measurement

cAMP was measured with a kit from BioVision (Mountain View, CA) according to the manufacturer's

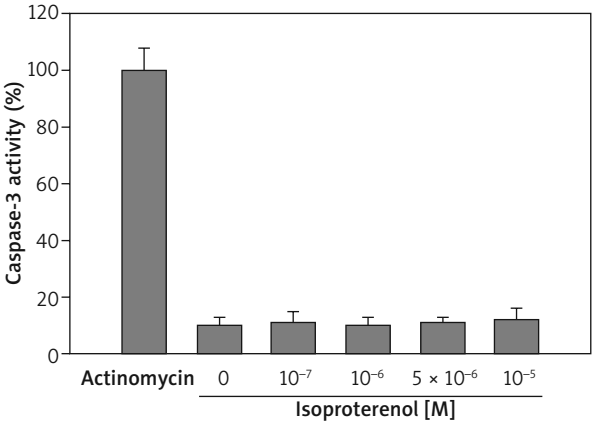

Figure 1. The caspase-3 activity was used to measure keratinocyte apoptosis. In order to acquire a positive control of apoptosis, keratinocytes were treated with actinomycin D

protocol. Samples were added to the 96-well plates, and incubations were treated with a cAMP antibody for 1 h at room temperature. cAMP conjugated with horseradish peroxidase was added to the sample mixture, and the immunoassay was handled according to the supplier's instructions. The reactions were accomplished after the addition of 1.0 N HCl. A450 nm was used to determine the reactions. Calculations depended on a standard curve for each experiment.

MTT assay of cellular proliferation

Keratinocytes were incubated in 96-well plates (5000 cells/well) for proliferation assays. After 24 h incubation in keratinocyte-SFM medium to make the cells attach,

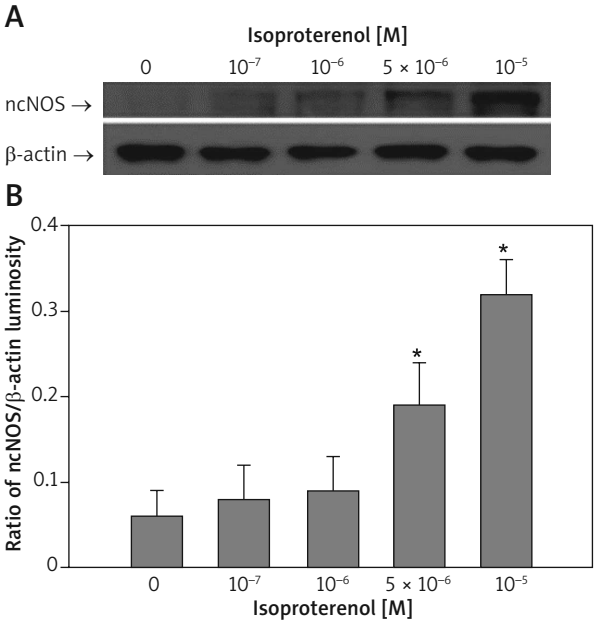

Figure 2. The expression of ncNOS protein in keratinocytes with isoproterenol exposure was analysed by Western blotting. *$P < 0.01$ vs. control and 10^{-7} M, and 10^{-6} M isoproterenol

the cells were processed for 48 h with various doses of experimental drugs and the cultures were terminated at 96 h. Cells were stained with MTT solution (5 mg/ml) for 4 h, the cells were then solubilized in 200 µl DMSO and absorbance readings were taken using microplate spectrophotometer (read absorbance at 570 nm).

Apoptosis test

The apoptosis test was determined using the Human active caspase-3 colorimetric assay kit (Invitrogen). The method was based on the measurement of the fluorescence in a sample after adding the specific caspase-3 antibody. The fluorescence elevated proportionally to the caspase-3 activity and could be quantified using a standard curve. After the keratinocytes were treated with various experimental conditions, they were washed 3 times with cold PBS and harvested. The cells were then treated as according to the manufacturer's protocol. In order to acquire a positive control of apoptosis, keratinocytes were treated with actinomycin D. The results are expressed in percent of the optical density at 450 nm measured.

Statistical analysis

All measurements are presented as means ± SD and were analysed using the statistical analysis system (SAS) application package. Differences among groups were determined by one-way analysis of variance. A difference with $p < 0.05$ was considered statistically significant.

Results

There was no apoptosis in keratinocytes with isoproterenol exposure

The caspase-3 activity was used to assess keratinocyte apoptosis in the study. Keratinocytes were treated with various isoproterenol concentrations (0 M, 10^{-7} M, 10^{-6} M, 5×10^{-6} M and 10^{-5} M). The caspase-3 activity did not change in control and experimental groups (Figure 1). As a result, various isoproterenol levels treatment did not result in apoptosis of keratinocytes.

The expression of iNOS and ncNOS protein in keratinocytes with isoproterenol exposure were analysed by Western blotting

The expression of iNOS and ncNOS protein in keratinocyte lysates after 24 h of culture with isoproterenol exposure were analysed by Western blotting. The Western blotting product bands for ncNOS and iNOS in protein lysates from isoproterenol-treated cells and control cells are shown in Figures 2 A and 3 A. The mean iNOS/β-actin and ncNOS/β-actin band intensity ratios are shown in Figures 2 B and 3 B. The mean ncNOS/β-actin and iNOS/β-actin band intensity ratios following isoproterenol treatment at doses of 5×10^{-6} M and 10^{-5} M were

significantly higher than those in the control group and isoproterenol treatment at 10^{-7} M and 10^{-6} M ($p < 0.01$). The mean iNOS/β-actin band intensity ratios following isoproterenol treatment at 10^{-5} M was significantly higher than isoproterenol treatment at 5×10^{-6} M ($p < 0.01$). The results showed that isoproterenol caused the presence of iNOS and ncNOS protein to raise.

The levels of nitric oxide in keratinocytes with treatment of different isoproterenol doses

The production of nitric oxide was assessed in keratinocytes with different levels of isoproterenol treatment.

The production of nitric oxide following isoproterenol treatment at 5×10^{-6} M and 10^{-5} M was significantly higher than in the control group, 10^{-7} M and 10^{-6} M treatment (Figure 4) ($p < 0.01$). The results showed that treatment of a higher isoproterenol dosage resulted in the production of nitric oxide increased in keratinocytes. The elevation of the production of nitric oxide should result from the presence of iNOS and ncNOS protein enhanced.

The production of cAMP in keratinocytes with treatment of different isoproterenol doses

The production of cAMP was measured in keratinocytes with treatment of different isoproterenol doses. The cAMP levels in the experimental group were significantly increased in comparison with the control group (Figure 5; $p < 0.01$). The cAMP levels in group 10^{-5} M was significantly increased in comparison with 5×10^{-6} M group, 10^{-6} M group and 10^{-7} M group ($p < 0.01$). Following an isoproterenol level increase, c-AMP production was significantly elevated.

MTT assay of cellular proliferation in keratinocytes with the treatment of various isoproterenol concentrations

Keratinocyte proliferation was measured by MTT assay. Keratinocyte proliferation of isoproterenol treatment at doses of 5×10^{-6} M and 10^{-5} M were significantly lower than in the control group, 10^{-7} M and 10^{-6} M groups (Figure 6). The results showed that treatment of higher isoproterenol dosage resulted in keratinocyte proliferation decreased.

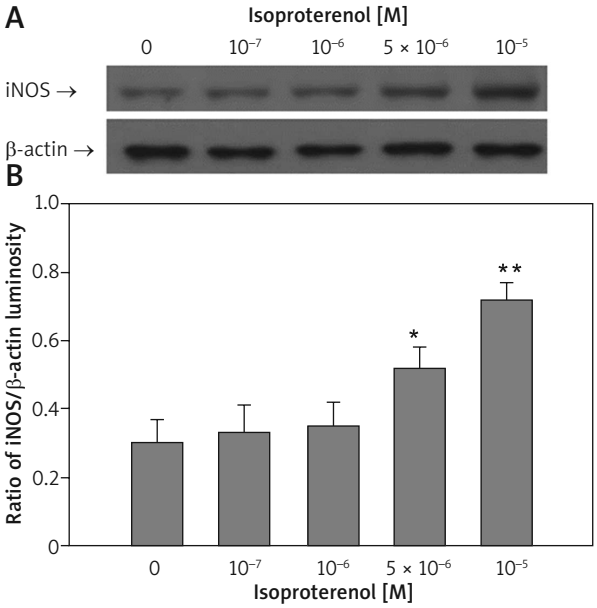

Figure 3. The expression of iNOS protein in keratinocytes with isoproterenol exposure was analysed by Western blotting. *$P < 0.01$ vs. control and 10^{-7} M, and 10^{-6} M isoproterenol. **$P < 0.01$ vs. control and 10^{-7} M, 10^{-6} M and 5×10^{-6} M isoproterenol

There was no apoptosis in keratinocytes with the treatment of isoproterenol plus different L-NMMA dosages

The caspase-3 activity was used to assess keratinocyte apoptosis in the study. Keratinocytes were treated with 10^{-5} M isoproterenol plus L-NMMA at doses of 0 M,

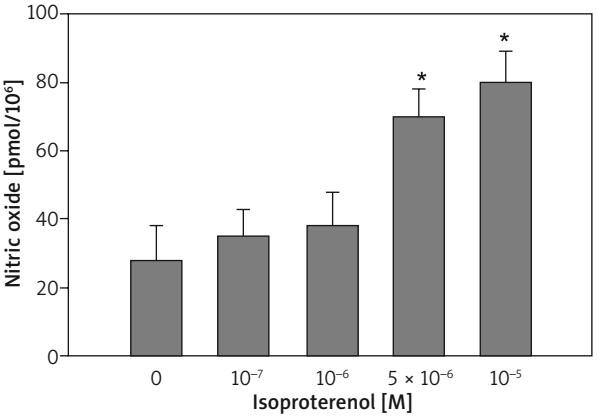

Figure 4. Effect of various doses of isoproterenol on NO release by human keratinocytes. *$P < 0.01$ vs. control, 10^{-7} M isoproterenol and 10^{-6} M isoproterenol

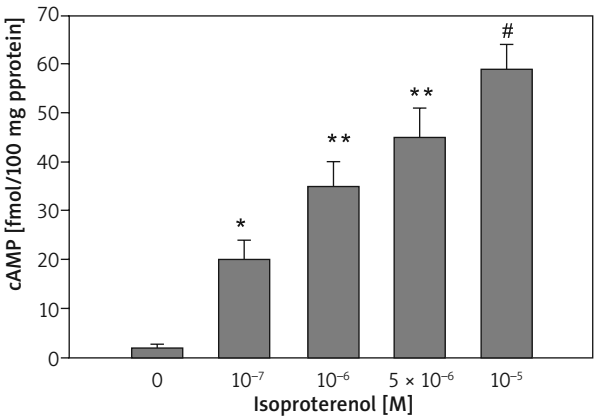

Figure 5. cAMP in keratinocytes with treatment of various isoproterenol doses. *$P < 0.01$ vs. control. **$P < 0.01$ vs. control and 10^{-7} M isoproterenol. ##$P < 0.01$ vs. control and 10^{-7} M, 10^{-6} M and 5×10^{-6} M isoproterenol

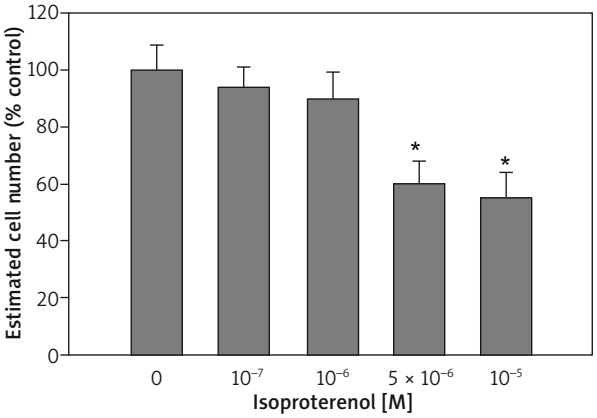

Figure 6. MTT assay of cellular proliferation in keratinocytes with the treatment of various isoproterenol concentrations. *P < 0.01 vs. control. **P < 0.01 vs. control and 10^{-7} M isoproterenol. ##P < 0.01 vs. control and 10^{-7} M, 10^{-6} M and 5×10^{-6} M isoproterenol

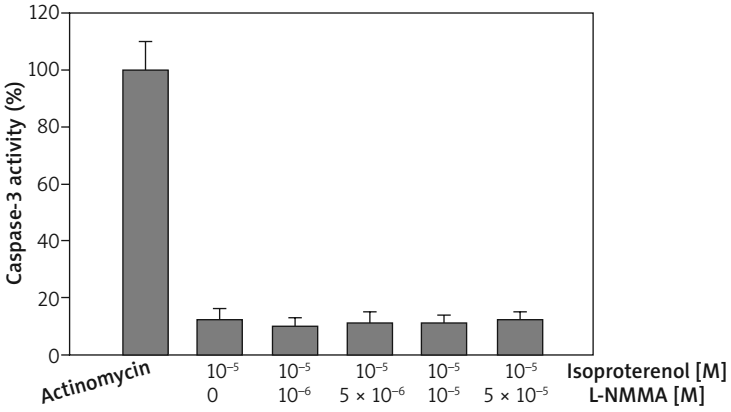

Figure 7. The caspase-3 activity was used to determine apoptosis in keratinocytes with the treatment of isoproterenol and various L-NMMA dosages. In order to acquire a positive control of apoptosis, keratinocytes were treated with actinomycin D

10^{-6} M, 5×10^{-6} M, 10^{-5} M and 5×10^{-5} M, respectively. The caspase-3 activity did not change in these experimental groups (Figure 7). In the study, isoproterenol plus different L-NMMA dosages treatment did not result in apoptosis of keratinocytes.

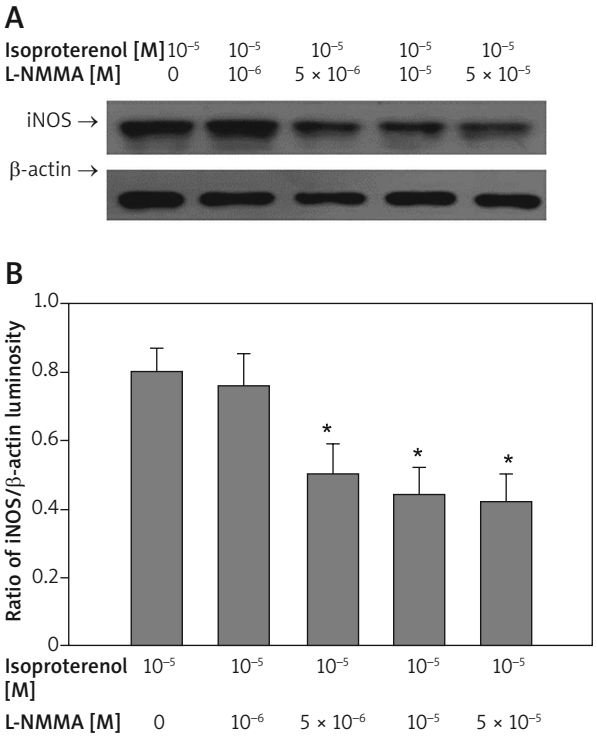

Figure 8. The expression of iNOS in keratinocytes with isoproterenol plus various L-NMMA level treatment was analysed by Western blotting. *P < 0.01 vs. 10^{-5} M isoproterenol and 10^{-5} M isoproterenol plus 10^{-6}M L-NMMA

The expression of iNOS and ncNOS protein in keratinocytes with isoproterenol plus different L-NMMA levels treatment was analysed by Western blotting

The Western blotting product bands for ncNOS and iNOS in protein lysates from isoproterenol plus L-NMMA treated cells were shown in Figures 8 A and 9 A. The mean iNOS/β-actin and ncNOS/β-actin band intensity ratios were shown in Figures 8 B and 9 B. The mean ncNOS/β-actin and iNOS/β-actin band intensity ratios following 10^{-5} M isoproterenol plus L-NMMA treatment at doses of 5×10^{-6} M, 10^{-5} M and 5×10^{-5} M were significantly lower than those following 10^{-5} M isoproterenol plus L-NMMA treatment at 0 M and 10^{-6} M (p < 0.01). The results showed that L-NMMA could block the expression of iNOS and ncNOS proteins caused by isoprenaline.

The levels of nitric oxide in keratinocytes with treatment of isoproterenol plus various L-NMMA levels

The production of nitric oxide following 10^{-5} M isoproterenol plus L-NMMA levels treatment at 10^{-6} M, 5×10^{-6} M, 10^{-5} M and 5×10^{-5} M was significantly lower than 10^{-5} M isoproterenol treatment (Figure 10) (p < 0.01). The results showed that L-NMMA could block the presence of NO caused by isoproterenol. The reduction in the production of nitric oxide should result from the presence of iNOS and ncNOS protein inhibited by L-NMMA.

The production of cAMP in keratinocytes with treatment of isoproterenol plus various L-NMMA levels

The cAMP levels did not significantly vary in all groups (Figure 11; p < 0.01). The results showed that L-NMMA·did not influence the production of c-AMP in keratinocytes with isoproterenol treatment.

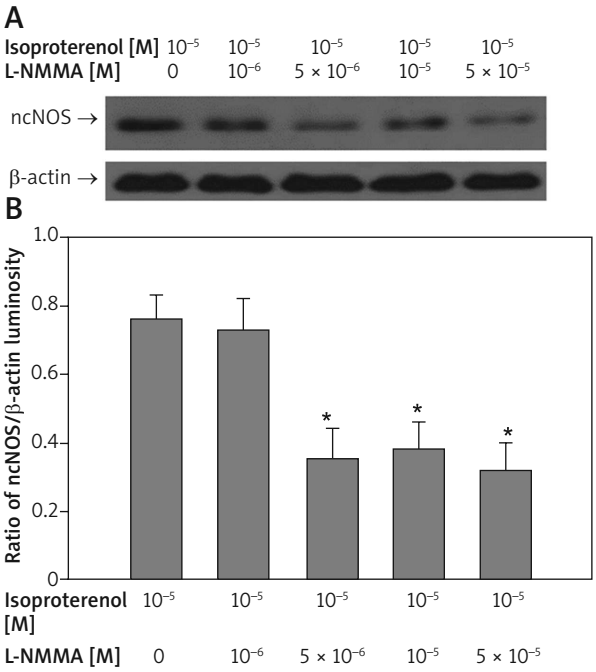

Figure 9. The expression of ncNOS in keratinocytes with isoproterenol plus various L-NMMA level treatment was analysed by Western blotting. *$P < 0.01$ vs. 10^{-5} M isoproterenol and 10^{-5} M isoproterenol plus 10^{-6}M L-NMMA

MTT assay of cellular proliferation in keratinocytes with treatment of isoproterenol plus various L-NMMA levels

Keratinocytes in three groups (10^{-5} M isoproterenol plus L-NMMA treatment at doses of 5×10^{-6} M, 10^{-5} M and 5×10^{-5} M) were significantly increased in comparison with two groups (10^{-5} M isoproterenol plus L-NMMA treatment at doses 0 M, 10^{-6} M; Figure 12; $p < 0.01$). The results showed that L-NMMA could block isoproterenol on proliferation inhibition.

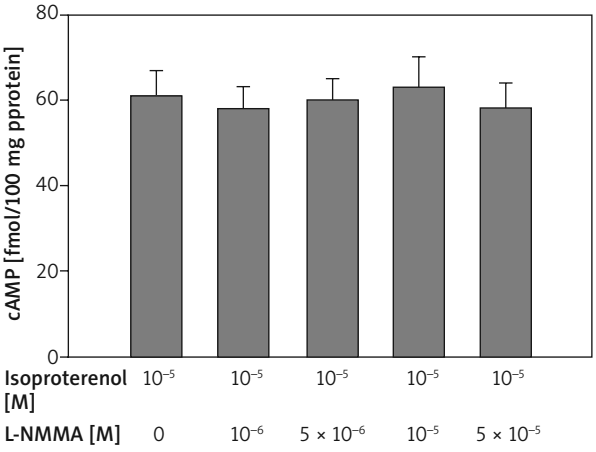

Figure 11. cAMP in keratinocytes with treatment of isoproterenol plus various L-NMMA levels

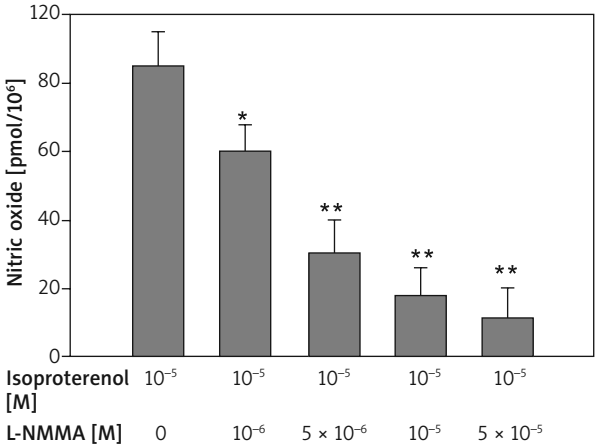

Figure 10. Effect of isoproterenol plus various L-NMMA doses on NO release by human keratinocytes. *$P < 0.01$ vs. 10^{-5} M isoproterenol. **$P < 0.01$ vs. 10^{-5} M isoproterenol and 10^{-5} M isoproterenol plus 10^{-6}M L-NMMA

Discussion

In the present study, we explored the possible role of β-ARs in the regulation of keratinocytes NOS activity and this may be important in β-AR-mediated inhibition of keratinocyte proliferation. Our study suggested the first direct evidence that β-ARs stimulation activated keratinocytes NOS to affect keratinocyte proliferation. Stimulation of β-ARs in keratinocytes with isoproterenol caused an increase in the expression of NOS and NO levels and reduced keratinocyte proliferation, a response that was blocked by treatment of L-NMMA. Our study demonstrates that β-ARs inhibited keratinocyte proliferation, and that they do so through generation of NO.

NO has been suggested to be a physiological modulator of cell proliferation, able to facilitate in many cases

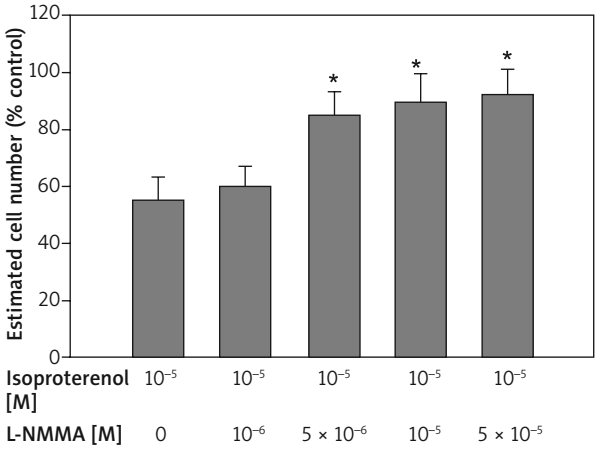

Figure 12. Cellular proliferation in keratinocytes with treatment of isoproterenol plus various L-NMMA levels. *$P < 0.01$ vs. 10^{-5} M isoproterenol and 10^{-5} M isoproterenol plus 10^{-6}M L-NMMA

the cell cycle arrest. NO donors with various chemical structures and mechanisms of action have been exerted to inhibit proliferation, without appearing significant cytotoxic effects, various cell types, including those obtained from various normal tissues and different tumours. The extensive expression of cell types suggests that the inhibitory activity of exogenously added NO indicates that the agent may have extensive anti-proliferative effects *in vivo* [16]. The expression of NOS and up-regulation of endogenous NO production mediated by interferon-γ (IFN-γ) or lipopolysaccharides (LPSs) [29–33] was demonstrated to inhibit the proliferation of cells, including vascular smooth muscle cells, liver stellate cells and pancreatic tumour cells [29, 33, 34]. Tumour necrosis factor-α (TNF-α) also stimulates the production of NO, inhibiting the proliferation of smooth muscle cells from blood vessels [32, 34, 35]. Apolipoprotein E is able to increase the expression of NOS in vascular smooth muscle cells, and inhibits its proliferation [36].

The important signal transduction mechanism for β-ARs implicated linking of the Gas protein to AC, which activated the conversion of adenosine triphosphate (ATP) to cAMP. cAMP then activated PKA. PKA was a protein kinase that aimed at several intracellular proteins, inducing a series of specific cellular reactions. For example, β2-ARs of vascular smooth muscle cell mediated vascular dilatation via an increase of cAMP, which activates PKA and results in smooth muscle cell relaxing [37]. Evidence for the relationship of the cAMP/PKA signalling pathway in β-AR-mediated NO generation mainly comes from endothelial cell studies. Voorhees *et al*. [20] reported that β-AR-mediated vascular relaxation is chiefly NO-dependent in isolated human umbilical vein. The NOS inhibitor (L-NMMA) blocked the relaxing reaction to β-AR stimulation, showing that the NO-dependent element of the β-AR relaxing reaction is regulated mainly through increment of cAMP.

Beta-ARs on human keratinocytes had been determined as being β2-ARs in type [38, 39], but the function of the receptor had not been completely clarified. Isoproterenol resulted in a concentration-dependent inhibition of keratinocyte proliferation through activation of β2-ARs. Several studies had shown that stimulation of keratinocyte β2-ARs increased intracellular cAMP and inhibited proliferation [1]. Activation of the cAMP-dependent pathway by direct activation of adenylate cyclase had been indicated to replicate the increment in intracellular calcium that was appeared with ligand stimulation of the β2-AR [40]. The elevation of the intracellular calcium concentration inhibited keratinocyte proliferation [41, 42]. In our study, we discovered another mechanism that β-ARs inhibited keratinocyte proliferation, and that they do so through generation of NO.

Conclusions

Much has been known about β-adrenergic control of skin homeostasis in the past few decades, and the more recent developing of NO as a biological signal transmission molecule has established an important new target for therapeutic application. In the last century, the connection between β-AR stimulation and NO production was investigated in detail, and the various control signal transmission cascades involved are starting to appear. Much work is required to fully explain the significance of this new mechanism of adrenergic control in integumentary physiology and pathophysiology, but it is obvious that the revelation of β-AR-NO coupling has revolutionized our comprehension of β-AR-mediated homeostatic control, and provides new possibilities in the therapy of integumentary disease conditions linked with the dysfunction of β-AR-mediated NO production.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

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