Peroxidated Squalene Induces the Production of Inflammatory Mediators in HaCaT Keratinocytes: A Possible Role in Acne Vulgaris

Monica Ottaviani[1,4], Theodosis Alestas[2,4], Enrica Flori[1], Arianna Mastrofrancesco[1], Christos C. Zouboulis[3] and Mauro Picardo[1]

In order to investigate whether products derived from the oxidation of sebum can be responsible for the induction of inflammatory processes, HaCaT keratinocytes were treated with peroxidated squalene. NF-κB activation, secretion, and expression of IL-6, as well as peroxisome proliferator-activated receptor alpha (PPARα) mRNA and protein levels, were measured at the end of the treatment and after 24 and 48 hours of recovery. Squalene peroxidation products were administered in amounts able to elicit significant hyperproliferation and to induce lipoxygenase (LOX) activity. The results showed an early activation of NF-κB followed by an increase in PPARα mRNA and protein levels. Moreover, squalene peroxides induced an initial upregulation of IL-6 production and secretion that was counteracted by PPARα activation, as suggested by the subsequent decrease of NF-κB nuclear translocation and IL-6 levels. Inflammatory processes play an important role in the development of acne vulgaris. In combination with our own previous findings, which indicated an association between LOX stimulation and increased percentage of proinflammatory lipids in acne as well as a correlation between increased cytokine levels in the infundibulum, pilosebaceous duct hyperkeratinization, and augmented sebogenesis, the present data further support the involvement of lipid peroxides, in particular squalene peroxides, in establishing an inflammatory process in acne.

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INTRODUCTION

Sebum is a holocrine product of human sebocytes partially constituted of a complex mixture of lipids. Owing to the presence of unsaturated lipids, sebum may undergo a process of peroxidation under the action of physical, chemical, and biological agents giving rise to proinflammatory lipidic by-products (Nazzaro-Porro *et al.*, 1986).

Squalene is a characteristic human sebaceous lipid. The presence of six double bonds allows squalene to easily undergo a photo-oxidation process giving rise to squalene mono-hydroperoxide as the principal product (Kohno *et al.*, 1993) together with several by-products (Picardo *et al.*, 1991a). Squalene peroxides (peroxidated squalene (P-Sq)) have been demonstrated to be involved in the pathogenesis of certain skin conditions (Ohkido *et al.*, 1980; Ohsawa *et al.*, 1984; Nazzaro-Porro *et al.*, 1987; Picardo *et al.*, 1991b) and to exert a comedogenic effect with a reaction degree higher than that of other peroxides (Motoyoshi, 1983; Chiba *et al.*, 2000). Moreover lipids from comedones in acne patients seem to be enriched in P-Sq, indicating a possible role of this peroxides in the development of acne lesions (Saint-Leger *et al.*, 1986a).

Acne is a disease of the pilosebaceous unit affecting – at different degrees of severity – about 85% of adolescents. Its pathogenesis is complex and is dependent on the interplay of multiple factors such as genetic predisposition, excess of sebum production, regulation of peroxisome proliferator-activated receptors (PPARs), abnormal follicular proliferation, and development of inflammation (Zouboulis *et al.*, 2005a, b). The sequence of events has not been elucidated yet, but current data indicate that inflammation could be a primary triggering factor and that *Propionibacterium acnes* is not the cause of initial acne lesions but a significant contributing element to the inflammatory stages of the disease (Zouboulis, 2001; Farrar and Ingham, 2004; Zouboulis *et al.*, 2005a).

Although excess of sebum excretion is involved in the pathophysiology of acne (Zouboulis, 2004), it is still unclear whether it is associated with the severity of the disease (Pochi

[1]San Gallicano Dermatological Institute IRCCS, Rome, Italy; [2]Department of Dermatology, Charité Universitaetsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany and [3]Departments of Dermatology and Immunology, Dessau Medical Center, Dessau, Germany

[4]These authors contributed equally to this work

Correspondence: Dr Mauro Picardo, San Gallicano Dermatological Institute IRCCS, Via Elio Chianesi 53, 00144 Rome, Italy. E-mail: picardo@ifo.it

Abbreviations: GC–MS, gas chromatography–mass spectrometry; LA, linoleic acid; LOX, lipoxygenase; LTB4, leukotriene B4; MTT, 3-(4,5-dimethyl-thiazolyl-2)-2,5-diphenyltetrazolium bromide; PPARα, peroxisome proliferator-activated receptor alpha; P-Sq, peroxidated squalene; Sq, squalene

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and Strauss, 1964; Cunliffe and Shuster, 1969; Burton and Shuster, 1971; Cunliffe, 1989; Youn *et al.*, 2005). Changes in secretion rates and composition of sebum seem to be associated with hyperproliferation of the keratinocytes lining the follicle wall during comedogenesis (Downing *et al.*, 1986). On the other hand, the quality of lipids and not the quantity has currently been addressed as a probable initiator of inflammatory processes (Cordain, 2005; Zouboulis *et al.*, 2005a).

Lipoxygenases (LOXs) are enzymes involved in the lipoperoxide formation, and the products of their action are thought to participate in the development of inflammatory skin lesions also by inducing keratinocytes hyperproliferation, such as psoriasis and atopic dermatitis (Iversen *et al.*, 1997; Yokomizo *et al.*, 2001; Haeggstrom and Wetterholm, 2002). Recent evidence indicates a role for LOX products, such as leukotrienes B4 (LTB4), in the development of inflammatory acne lesions (Zouboulis, 2003; Alestas *et al.*, 2006). Preliminary clinical studies have shown that a specific inhibitor of 5-LOX reduces the number of inflammatory lesions and the levels of sebum peroxides in acne patients, supporting the hypothesis that lipoperoxides may exert a proinflammatory action on the pilosebaceous unit (Zouboulis *et al.*, 2003, 2005b).

The aim of this study was to investigate the possible role of P-Sq in the initiation and development of inflammatory events using a human keratinocytes cell line (HaCaT) as model. We evaluated the effect of P-Sq on HaCaT keratinocytes proliferation and LOX activity. Among the inflammatory pathways, we focused on the activation of NF-κB, which coordinates the expression of different proinflammatory genes, synthesis and secretion of the cytokine IL-6, and induction of PPARα. The latter inhibits the synthesis of proinflammatory molecules, such as IL-6, via a decreased activity of the NF-κB signalling pathway (Poynter and Daynes, 1998; Staels *et al.*, 1998; Delerive *et al.*, 1999, 2001; Marx *et al.*, 1999), and it is likely to exhibit a role in acne (Zouboulis *et al.*, 2005b; Alestas *et al.*, 2006). We could show that P-Sq stimulates HaCaT keratinocytes proliferation, LOX activity, and NF-κB activation with the consequent secretion of IL-6. Finally, P-Sq induces mRNA and protein PPARα expression that leads to NF-κB inhibition and decreased IL-6 levels. These results indicate a direct involvement of P-Sq in the onset of inflammatory state. Considering that Sq is a specific human sebaceous product and that its peroxidation products exert a comedogenic effect, our own previous data indicate the involvement of P-Sq in the development of inflammation in acne.

RESULTS

Proliferative effect of P-Sq on HaCaT keratinocytes

In order to evaluate a possible role of P-Sq on the hyperkeratinization of the pilosebaceous duct, we focused on the proliferation response of HaCaT keratinocytes after treatment with P-Sq in a concentration range from 0.1 to 10 μg/ml by means of the 3-(4,5-dimethyl-thiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) test and cell counting. P-Sq exerted different effects on HaCaT cell proliferation depending on the concentration administered. In particular, concentrations below 0.75 μg/ml did not affect HaCaT keratinocytes proliferation, whereas concentrations between 0.75 and 5.0 μg/ml induced a significant proliferative stimulus (approximately 30%, $P<0.05$) and higher concentrations began to have a toxic effect proportional to P-Sq dose (Figure 1a). Cell counting confirmed the results obtained with the MTT assay and indicated a dose-dependent effect of P-Sq ($R^2=0.92$) (Figure 1b). Sq treatment (0.1–5 μg/ml) did not affect HaCaT keratinocytes proliferation and resulted in a toxic effect at higher concentrations (Figure 1a). These data suggest that P-Sq may be involved in the induction of the hyperkeratosis of the pilosebaceous duct.

Induction of LOX activity in HaCaT keratinocytes by P-Sq

Our own previous data indicated that lipoperoxides and sebum production could be related to 5-LOX activity in acne patients (Zouboulis *et al.*, 2003, 2005a, b). In order to investigate whether P-Sq could stimulate LOX activity, we evaluated linoleic acid (LA) consumption in control- and P-Sq-treated HaCaT keratinocytes by gas chromatography–mass spectrometry (GC–MS). Cells treated with P-Sq consumed approximately 30% more LA than untreated ones (Figure 2). The experiment was further conducted by preincubating the cells with 5,8,11,14-eicosatetraynoic acid, a broad LOX inhibitor that completely abrogated LA cell consumption by

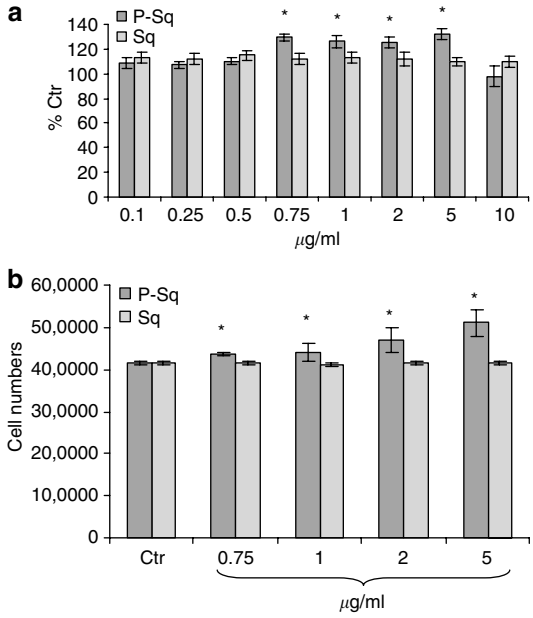

Figure 1. P-Sq exerts a proliferative effect on HaCaT keratinocytes. Keratinocytes were treated with P-Sq and Sq (0.1–10 μg/ml) for 3 hours in serum-free conditions, incubated with an MTT solution for 2 hours at 37°C, and lysed by dimethylsulfoxide. All values are related to control ones derived from untreated cells. (**a**) The data are reported as percentage of absorbance at 570 nm in comparison with the control. (**b**) Cell proliferation was determined also by the Trypan blue exclusion test. After treatment, HaCaT keratinocytes were harvested and counted using a hemocytometer. The shown values are the mean ± SD of three experiments in triplicate. Statistical differences were evaluated using Mann–Whitney test (*$P<0.05$ with respect to untreated cells).

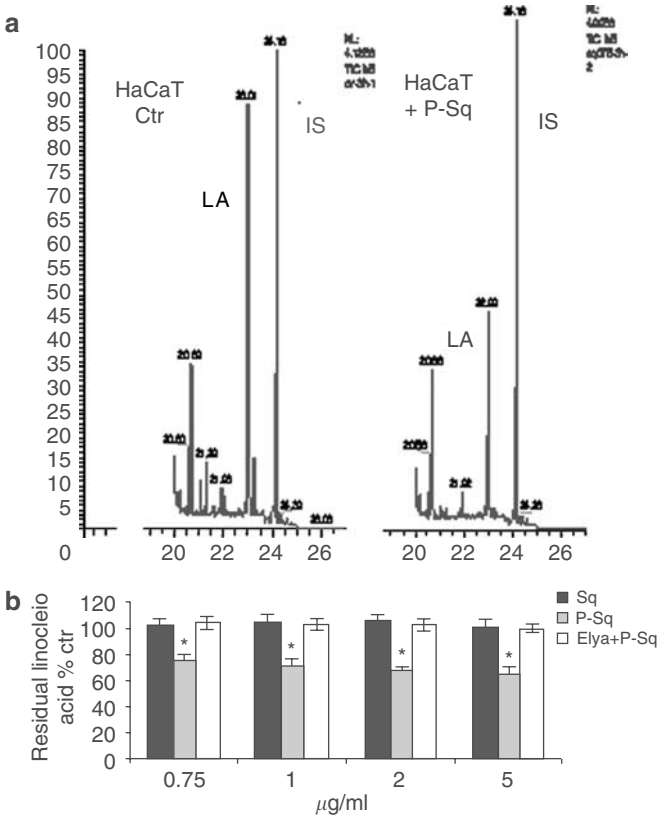

Figure 2. P-Sq is able to induce HaCaT LOX activity. Directly or after a pretreatment with 5,8,11,14-eicosatetraynoic acid (10 μM), HaCaT keratinocytes were treated with P-Sq and Sq (0.75–5.0 μg/ml) for 3 hours in serum-free conditions. LA was added to the cell lysates and, after the incubation at 37°C for 12 minutes, its amount was evaluated by GC–MS analysis. (**a**) A representative chromatogram is shown. Nonadecanoic acid was used as an internal standard (IS). (**b**) LA recovery presentation. The results are expressed as percentage of residual LA after the incubation in comparison with untreated cells. The values shown are mean ± SD representative of three experiments in duplicate. Statistical differences were evaluated using the Mann–Whitney test (*$P<0.02$ with respect to untreated cells, °$P<0.02$ with respect to P-Sq-treated cells).

HaCaT keratinocytes (Figure 2). These results demonstate that P-Sq stimulates LOX activity leading to the generation of proinflammatory mediators.

Induction of NF-κB activation

To investigate the potential effect of P-Sq on the induction of inflammatory response in HaCaT keratinocytes, NF-κB activation was determined by FACS analysis in control- and P-Sq-treated cells. A significant increase in NF-κB activation was observed in P-Sq-treated HaCaT keratinocytes, when compared to controls, just after 1 hour of treatment (Figure 3). This induction was maintained at an elevated level up to 2 hours after P-Sq withdrawal, reaching again the basal level after 24 hours. Sq did not activate the NF-κB pathway. Considering that the majority of the proinflammatory genes are under the control of the NF-κB signalling pathway, it is likely that P-Sq can induce the production of inflammatory mediators in HaCaT keratinocytes.

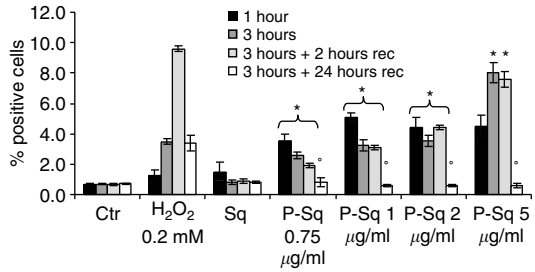

Figure 3. P-Sq induces NF-κB nuclear translocation in HaCaT keratinocytes. Keratinocytes were treated with P-Sq and Sq (0.75–5.0 μg/ml) for 1 and 3 hours under serum-free conditions. Nuclei isolation was performed by the Cycle TEST™ PLUS DNA Reagent kit immediately after the treatment and 2 and 24 hours after the P-Sq and Sq depletion. NF-κB was detected by a mAb and analyzed by flow cytometer. Results are expressed as percentage of positive cells and represent the mean ± SD of three experiments in duplicate. Statistical differences were evaluated using the Mann–Whitney test (*$P<0.01$ with respect to untreated control, **$P<0.005$ with respect to untreated control, °$P<0.01$ with respect to cells treated with P-Sq for 1 hour).

Induction of IL-6 expression and secretion in HaCaT keratinocytes by P-Sq

Among keratinocyte-derived cytokines and chemokines, the proinflammatory ones IL-1, IL-6, IL-8, and tumor necrosis factor-α are best characterized (Grone, 2001). In order to verify whether P-Sq treatment can be responsible for the development of inflammatory reaction in keratinocytes, we focused our interest on IL-6 induction and secretion. We observed, by means of fluorimetric analysis, an increase in the release of IL-6 in HaCaT keratinocytes treated with P-sq at a concentration of 1, 2, and 5 μg/ml for 24 hours after P-Sq removal (control: 200 ± 6 pg/ml; P-Sq 1 μg/ml: 262 ± 12 pg/ml; P-Sq 2 μg/ml: 262 ± 13 pg/ml; P-Sq 5 μg/ml: 293 ± 15 pg/ml; $P<0.05$). Cytokine release began to decrease 48 hours after treatment (P-Sq 1 μg/ml: 238 ± 13 pg/ml; P-Sq 2 μg/ml: 239 ± 15 pg/ml; P-Sq 5 μg/ml: 243 ± 16 pg/ml; $P<0.05$) (Figure 4). The evaluation of IL-6 mRNA levels confirmed the results derived from the protein studies. An upregulation of IL-6 mRNA levels in a dose-dependent manner was observed in cells treated with P-Sq at 1, 2, and 5 μg/ml compared to controls, immediately after the treatment (P-Sq 1 μg/ml: 109% ± 3; P-Sq 2 μg/ml: 118% ± 4; P-Sq 5 μg/ml: 133% ± 5; $P<0.05$; $R^2>0.9$). The level of IL-6 gene expression in treated cells remained steady up to 24 hours after P-Sq removal (P-Sq 1 μg/ml: 113% ± 3; P-Sq 2 μg/ml: 119% ± 3; P-Sq 5 μg/ml: 136% ± 4; $P<0.05$), returning again to the control level after 48 hours (P-Sq 1 μg/ml: 104% ± 1; P-Sq 2 μg/ml: 106% ± 1; P-Sq 5 μg/ml: 111% ± 1; $P<0.05$) (Figure 5). P-Sq at 0.75 μg/ml and Sq did not affect IL-6 expression and secretion (Figures 4 and 5). The results obtained demonstrate that P-Sq is able to induce an inflammatory stimulus in HaCaT keratinocytes by increasing IL-6 expression and secretion.

Induction of PPARα mRNA and protein expression in HaCaT keratinocytes by P-Sq

In order to investigate the machinery of the inflammatory response more thoroughly, we evaluated the P-Sq effect on

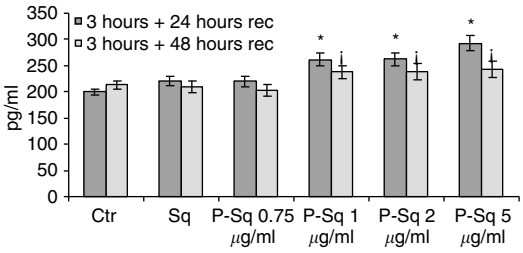

Figure 4. P-Sq induces an upregulation of HaCaT keratinocytes IL-6 secretion. HaCaT keratinocytes were treated with P-Sq and Sq (0.75–5.0 μg/ml) for 3 hours under serum-free conditions and collected 24 and 48 hours after P-Sq and Sq depletion. IL-6 release was determined by Fluorokine® MAP cytokine multiplex kits using a Bioplex reader. All values are related to control ones derived from untreated cells. Results are expressed as average mean of protein concentration (pg/ml) ± SD and represent the mean of three experiments in duplicate. Statistical differences were evaluated using the Mann–Whitney test (*$P<0.05$ with respect to untreated control, °$P<0.05$ with respect to treated cells 24 hours after P-Sq removal).

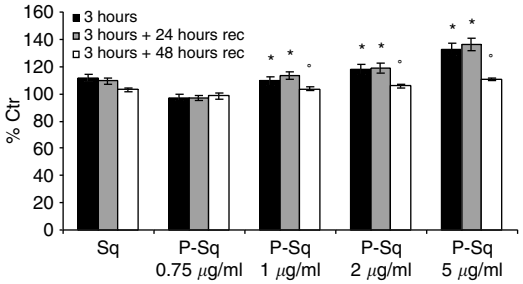

Figure 5. P-Sq is able to upregulate IL-6 mRNA expression in HaCaT keratinocytes. HaCaT keratinocytes were treated with P-Sq and Sq (0.75–5.0 μg/ml) for 3 hours in serum-free conditions and collected 24 and 48 hours after P-Sq and Sq depletion. For each sample, the same amount (1 μg) of total mRNA was reverse transcribed into cDNA and amplified by PCR reaction. The PCR products were visualized on agarose gel and the band intensities were evaluated by densitometric analysis. The values shown represent mean ± SD of three experiments. Statistical differences were evaluated using the Mann–Whitney test (*$P<0.05$ with respect to untreated control, °$P<0.05$ with respect to treated cells after P-Sq removal).

mRNA expression and protein synthesis of PPARα, which plays an important role in the inflammatory cascade (Delerive *et al.*, 2001). Immediately after treatment, P-Sq at 1, 2, and 5 μg/ml caused an upregulation of PPARα mRNA levels compared to controls (P-Sq 1 μg/ml: 138% ± 2; P-Sq 2 μg/ml: 142% ± 4, P-Sq 5 μg/ml: 150% ± 4; $P<0.05$). Afterwards, the level of PPARα gene expression in the treated cells decreased during the following 48 hours. Conversely, the treatment of HaCaT keratinocytes with P-Sq at 0.75 μg/ml or Sq did not exert any effect on the expression pattern of PPARα, compared to untreated controls (Figure 6). Significant upregulation of PPARα protein levels was observed by means of Western blot analysis in cells treated with P-Sq at 1, 2, and 5 μg/ml compared to controls, 24 hours after P-Sq removal (P-Sq 1 μg/ml: 130% ± 3; P-Sq 2 μg/ml: 135% ± 4; P-Sq 5 μg/ml: 154% ± 4; $P<0.05$). Sq did not exert any effect on PPARα protein levels (Figure 7). The PPARα upregulation supports

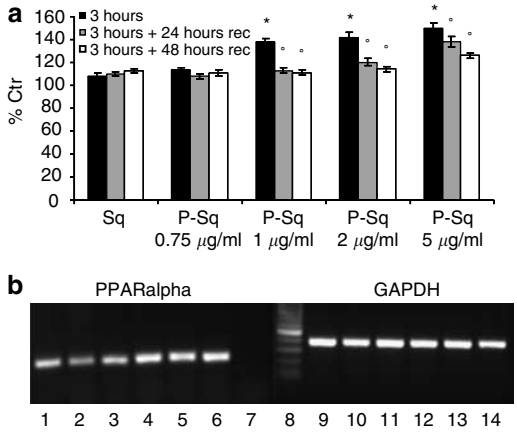

Figure 6. P-Sq induces an upregulation of PPAR-α mRNA level in HaCaT keratinocytes. HaCaT keratinocytes were treated with P-Sq and Sq (0.75–5.0 μg/ml) for 3 hours under serum-free conditions and collected at the end of the treatment and 24 and 48 hours after P-Sq and Sq depletion. For each sample, the same amount (1 μg) of total mRNA was reverse transcribed into cDNA and amplified by PCR reaction. The PCR products were visualized on agarose gel and the band intensities were evaluated by densitometric analysis. The values shown represent mean ± SD of three experiments. (**a**) Statistical differences were evaluated using the Mann–Whitney test (*$P<0.05$ with respect to untreated control, °$P<0.05$ with respect to treated cells after P-Sq removal). (**b**) PCR amplification of PPAR-α representative data is shown. Lanes 1–9: untreated HaCaT; lanes 2–10: HaCaT treated with Sq; lanes 3 and 11: HaCaT treated with P-Sq 0.75 μg/ml for 3 hours; lanes 4 and 12: HaCaT treated with P-Sq 1 μg/ml for 3 hours; lanes 5 and 13: HaCaT treated with P-Sq 2 μg/ml for 3 hours; lanes 6 and 14: HaCaT treated with P-Sq 5 μg/ml; lane 7: negative control; lane 8: molecular weight marker, 10 μl of 100 bp DNA ladder (Promega).

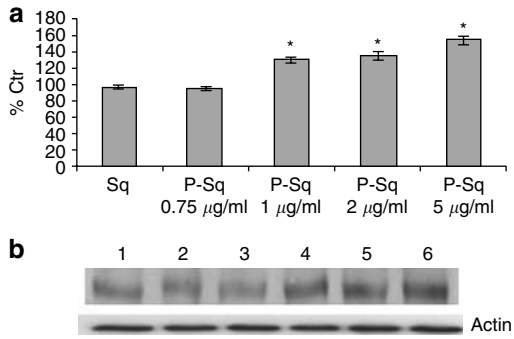

Figure 7. P-Sq induces an upregulation of PPARα protein levels in HaCaT keratinocytes. HaCaT keratinocytes were treated with P-Sq and Sq (0.75–5.0 μg/ml) for 3 hours in serum-free conditions and collected 24 hours after P-Sq and Sq depletion. Twenty micrograms of total cell lysate proteins was resolved under reducing conditions and transferred to nitrocellulose membrane. The membrane was incubated for 1 hour at room temperature, with a monoclonal antibody against PPARα (clone 3B6). As secondary antibody, goat anti-mouse IgG-HRP was used, followed by ECL detection. The band intensities were evaluated by densitometric analysis. The values shown represent the mean ± SD of three experiments. (**a**) Statistical differences were evaluated using the Mann–Whitney test (*$P<0.05$ with respect to untreated control). (**b**) Representative blot is shown. Lane 1: untreated HaCaT; lane 2: HaCaT treated with Sq; lane 3: HaCaT treated with P-Sq 0.75 μg/ml for 3 hours; lane 4: HaCaT treated with P-Sq 1 μg/ml for 3 hours; lane 5: HaCaT treated with P-Sq 2 μg/ml for 3 hours; lane 6: HaCaT treated with P-Sq 5 μg/ml for 3 hours.

the data mentioned above, which highlighted the possible induction of inflammatory response in HaCaT keratinocytes after the treatment with P-Sq.

DISCUSSION

In this study, we investigated the potential role of photo-oxidation products of Sq (P-Sq), a characteristic sebaceous lipid, in the induction of inflammatory mediators.

Treatment of the human keratinocyte cell line HaCaT with P-Sq in a certain concentration range stimulated cell proliferation and LOX activity. P-Sq treatment also induced enhancement of NF-κB nuclear translocation followed by an increase in the expression and secretion of the proinflammatory cytokine IL-6 and enhancement of PPARα mRNA and protein levels.

Sebaceous duct keratinocytes from comedones exhibit a hyperproliferative response compared to normal keratinocytes (Knaggs *et al.*, 1994; Huges *et al.*, 1996). The hyperproliferative behavior of HaCaT keratinocytes induced by P-Sq could support the idea that different sebum compositions may be involved in comedone formation and, in particular, that Sq peroxides could be – at least partially – responsible for this event. P-Sq seems to be involved in early stages of the inflammatory response and it also probably plays an important role in sustaining it through activation of LOX. HaCaT keratinocytes treated with Sq peroxide at concentrations inducing hyperproliferation exhibited increased LOX activity. LOX are enzymes involved in the lipoperoxide formation and their products are suggested to participate in the development of inflammatory skin diseases with keratinocyte hyperproliferation, such as psoriasis and atopic dermatitis (Iversen *et al.*, 1997; Yokomizo *et al.*, 2001; Haeggstrom and Wetterholm, 2002). A LOX involvement in the pathogenesis of acne has recently been suggested, in particular LTB4 synthesis through 5-LOX (Zouboulis, 2001; Zouboulis, 2003; Alestas *et al.*, 2006). LTB4 is a potent chemoattractant agent being able to recruit neutrophils and macrophages, and its production could justify the initial neutrophil infiltration described in acne leading to inflammatory lesions also in the absence of *P. acnes* (Yokomizo *et al.*, 2001; Jeremy *et al.*, 2003). LTB4 synthesis is increased by inflammatory mediators and LTB4 itself stimulates the production of a number of proinflammatory cytokines, indicating its ability to increase and prolong the inflammatory stage (Crooks and Stockley, 1998). The presence of an inflammatory stimulus after treatment with P-Sq was demonstrated by the concentration-dependent activation of NF-κB with the consequent increase in the expression and secretion of IL-6. We focused on IL-6 because it is one of the best-characterized proinflammatory cytokines in keratinocytes, which is expressed under a variety of conditions (Grone, 2002). IL-6 stimulates keratinocyte proliferation (Grossman *et al.*, 1989; Sato *et al.*, 1999; Sugawara *et al.*, 2001) and its transcription is controlled by PPARα (Delerive *et al.*, 2001). PPARα exerts a modulatory role in the control of the inflammatory response (Devchand *et al.*, 1996; Delerive *et al.*, 2001; Kota *et al.*, 2004) by antagonizing NF-κB and AP-1 signalling pathways (Poynter and Daynes, 1998; Staels *et al.*, 1998; Delerive *et al.*, 1999; Marx *et al.*, 1999). After the treatment with P-Sq, HaCaT keratinocytes exhibited higher levels of PPARα transcripts probably as a feedback in anti-inflammatory response to the stimulus. Moreover, the fact that NF-κB activation and IL-6 values return to basal levels with the increase of PPARα expression allows us to speculate that it may be owing to PPARα action.

In conclusion, we demonstrated that squalene peroxide can induce an inflammatory response in keratinocytes through LOX activation and increase in the proinflammatory cytokine IL-6 production. Therefore, we suggest that the products of LOX activity may contribute to an implementation of the inflammatory reaction with a concomitant anti-inflammatory feedback response of (healthy) keratinocytes as demonstrated by the increase of PPARα mRNA and protein levels.

As shown before, systemic treatment with a 5-LOX inhibitor reduces the inflammatory lesions in acne patients with the concomitant decrease of sebum hydroperoxides amount, indicating that these compounds have a role in the pathogenesis of acne exerting proinflammatory activity on the pilosebaceous unit (Zouboulis, 2003). Moreover, previously published *in vivo* study demonstrated that skin surface lipids from acne patients, in comparison to healthy subjects, were enriched in polar lipids that appeared to be derived mainly from Sq oxidation as confirmed by *in vitro* study (Saint-Leger *et al.*, 1986a, b). These and our present findings allow us to suggest a possible role of P-Sq in the development of acne lesions.

MATERIALS AND METHODS

Cell culture

The spontaneously immortalized human keratinocyte cell line (HaCaT) (Boukamp *et al.*, 1988) was cultured in DMEM (Euroclone, Wetherby, West York, UK), supplemented with fetal bovine serum (10%), L-glutamine (2 mM), penicillin (100 μg/ml), and streptomycin (100 μg/ml) (all from GIBCO, Milan, Italy) at 37°C in 5% CO_2/95% air atmosphere in a humidified incubator.

Sq peroxidation

Squalene (1 mg/ml) in hexane was UVA irradiated (365 nm) using a UVA lamp with an output of 17.6 mW/cm^2 for 1 hour at a distance of 30 cm (Psorisan® 900, Dr Hönle Medizintechnik, Munich, Germany). The amount of P-Sq was determined by a Lambda 25 UV/Vis spectophotometer (Perkin-Elmer, Bucks, UK) according to a method using dietyl-para-phenilendiamine (DEPD) as reagent (Nazzaro-Porro *et al.*, 1986).

Treatments

Cell proliferation. HaCaT keratinocytes were seeded in 24-well plates (2×10^4 cells per well) and treated in serum-free conditions for 3 hours with Sq or P-Sq at concentrations of 0.1, 0.25, 0.5, 0.75, 1, 2, 5, and 10 μg/ml. At the end of the treatment, fresh medium was added and cell proliferation was determined after 24 hours.

LOX activity. HaCaT keratinocytes were seeded in 25 cm^2 culture flasks (5×10^5 cells per flask) and treated in serum-free conditions with Sq or P-Sq (0.75, 1, 2, and 5 μg/ml) for 3 hours. In a part of the

experiments, cells were pretreated for 48 hours with 5,8,11,14-eicosatetraynoic acid (10 μM) (Sigma-Aldrich, Milan, Italy). Cells were analyzed at the end of the treatments or after 24 hours from the addition of fresh medium.

NF-κB activation. HaCaT keratinocytes were seeded in six-well plates (2×10^5 cells per well) and treated in serum-free conditions with Sq or P-Sq (0.75, 1, 2, and 5 μg/ml) for 1 and 3 hours, respectively. Cells were analyzed at the end of the treatments or after 24 hours from the addition of fresh medium.

IL-6 and PPARα mRNA expression. HaCaT keratinocytes were seeded in 75 cm^2 culture flasks (2×10^6 cells per flask) and treated in serum-free conditions with Sq or P-Sq (0.75, 1, 2, and 5 μg/ml) for 3 hours. Cells were collected at the end of the treatment or after 24 and 48 hours from the addition of fresh medium.

PPARα protein expression. HaCaT keratinocytes were seeded in 75 cm^2 culture flasks (2×10^6 cells per flask) and treated in serum-free conditions with Sq or P-Sq (0.75, 1, 2, and 5 μg/ml) for 3 hours. Cells were collected after 24 and 48 hours from the addition of fresh medium.

IL-6 release. HaCaT keratinocytes were seeded in 24-well plates (2×10^4 cells per well) and treated in serum-free conditions with Sq and P-Sq (0.75, 1, 2, and 5 μg/ml) for 3 hours. At the end of the treatment, surnatants were removed, fresh medium was added, and free cell supernatants were collected after 24 and 48 hours.

Cell proliferation

Cell proliferation was evaluated by MTT assay (Sigma-Aldrich). Cells were incubated with an MTT solution (final concentration 0.5 mg/ml) for 2 hours at 37°C and lysed by dimethyl sulfoxide. Absorbance at 570 nm was measured by a spectrophotometer μQUANT (BIO-TEK). The results represent the mean of three experiments in triplicate. In addition, cell proliferation was also determined by the Trypan blue exclusion test. Cells were harvested by incubation in 0.5% trypsin and 0.2% EDTA for 10 minutes at 37°C, and counted using a hemocytometer. The results represent the mean of three experiments in triplicate.

LOX activity

LOX activity was evaluated by maintaining GC–MS the consumption of LA, the substrate of the enzyme, added to the cell lysates. Treated cells were lysed in TRIS-EDTA-EGTA (TEE) buffer (50 mM Tris/HCl pH 8.5, 1 mM EDTA (both from Merck, Darmstadt, Germany), 1 mM EGTA and 10 μg/ml leupepetin (both from Sigma-Aldrich)) by repeated freezing in liquid nitrogen and thawing. Protein concentration was determined in the supernatants according to Bradford (Bradford, 1976), using BSA as a standard, by a Lambda 25 UV/Vis spectophotometer (Perkin-Elmer, UK). Aliquots of supernatants containing 150 μg of proteins were diluted to 250 μl with TEE buffer, and were incubated with LA dissolved in 5 μl of ethanol (final concentration 100 μM) for 12 minutes at 37°C (Siebert *et al.*, 2001). The reaction was terminated by adding 40 μl of sodium formate buffer (1 M, pH 3.1) and 100 μl of nonadecanoic acid (100 μg/ml; both from Sigma-Aldrich) was added as internal standard for GC–MS analysis. Samples were extracted twice with ethyl acetate and the combined organic extracts were evaporated under N_2 flow. The samples were then methylated by adding 500 μl of diazomethane. After 30 minutes at room temperature, the samples were dried under N_2 flow, dissolved in 100 μl of ethyl acetate, and analyzed using a combined GC–MS system (Trace GC Ultra, Thermofinnigan equipped with a single quadrupole mass spectrometer Trace DSQ, Thermofinnigan) on capillary column (RTX-5MS 30 m × 0.25 mm × 0.25 μm, Restek, Bellefonte, PA). Helium was used as the carrier gas. An oven temperature gradient from 40 to 280°C at 10°C/minute was used. Mass spectra were recorded in Electronic Impact and in SIM modality (t_R(LA) = 23 minutes (m/z 81, 294) t_R(IS) = 24.10 minutes (m/z 143, 312)). The results were obtained after time integration of chromatogram and final reprocessing of the peak areas. They were reported as a percentage of residual LA. The results represent the mean of three experiments in triplicate.

NF-κB activation

HaCaT keratinocytes were trypsinized, centrifuged at 200 × *g*, resuspended in phosphate-buffered saline solution (PBS; Euroclone LTD, UK), and washed twice. Determination of NF-κB activation was performed using the Cycle TEST™ PLUS DNA Reagent Kit, according to the manufacturer's procedure (Becton-Dickinson, Mountain View, CA). Briefly, cells were resuspended in the buffer solution and centrifuged at 200 × *g* twice. Then, cells were incubated with anti-NF-κB p65 mouse monoclonal antibody (1:1,000 in PBS) for 10 minutes followed by goat anti-mouse IgG-FITC antibody (1:100 in PBS) (both from Santa Cruz Biotechnology, Italy). The samples were analyzed on a FACSCalibur flow cytometer (Becton-Dickinson) equipped with a 488 nm argon laser. A total of 1×10^4 cells from each sample were acquired, and CELL-QUEST software was used to analyze the data. H_2O_2 (0.2 mM) treatment was used as NF-κB activation-positive control. The results represent the mean of three experiments in triplicate.

IL-6 release

IL-6 release into the supernatants was assessed using the Fluorokine® MAP cytochine multiplex kits (R&D Systems, UK), designed to be used in a Bioplex™ reader (Bio-Rad, Milan, Italy). The assay was performed according to the manufacturer's instructions. The results represent the mean of three experiments in triplicate.

RNA isolation

Total RNA from HaCaT keratinocytes was isolated using the Trizol method (Invitrogen, Italy) according to the manufacturer's procedure, and stored at −80°C until use.

Reverse transcriptase-PCR

The oligonucleotide primers for PCR were synthetized by MWG-Biotech AG (Italy) and were as follows: glyceraldehyde-3-phosphate dehydrogenase sense: 5′-GCACCACCAACTGCTTAGC-3′ and antisense: 5′-TGCTCAGTGTAGCCCAGG-3′; IL-6 sense: 5′-TGAACCTT CCAAAGATGGCTG-3′ and antisense: 5′-ACATTTGCCGAAGAGCC CTC-3′; PPAR-α sense: 5′-TGTCGGGATGTCACACAACG-3′ and antisense: 5′-TGGTTCCATGTTGCCAAGAG-3′.

Reverse transcriptase-PCR was carried out using 1 μg of total RNA. After denaturation in diethylpyrocarbonate-treated water (Promega Corporation, Madison, WI) at 70°C for 10 minutes, RNA

was reverse transcribed into cDNA using ImProm-II™ Reverse Transcriptase (1 μl per reaction; Promega) and 0.5 μg of oligo(dT) primers, at 42°C for 60 minutes, in a total volume of 20 μl ImProm-II™ 5× Reaction Buffer (Promega), containing 1.5 mM $MgCl_2$, 0.5 mM dNTP, and 20 U RNase inhibitor. Reverse transcriptase was inactivated at 70°C for 15 minutes and the RNA template was digested by RNase H at 37°C for 30 minutes. In each case, samples containing no reverse transcriptase (negative control) were included to exclude amplification from contaminating DNA. PCR reaction was carried out in the PCR Master Mix buffer (25 μl total volume; Promega) containing 1 μl cDNA, 20 pmol of oligonucleotide primers, 1 U *Taq* DNA polymerase, 1.5 mM $MgCl_2$, and 0.2 mM dNTP. PCR amplification was performed with an iCycler thermal cycler (Bio-Rad). Each cycle consisted of 1 minute at 94°C, 1 minute at annealing temperature, and 1 minute at 72°C, for a total of 32 cycles. The final product was extended for 5 minutes at 72°C. In every case, PCR-negative control (without DNA) was included. PCR conditions were as follows (annealing temperature, amplicon length): glyceraldehyde-3-phosphate dehydrogenase: 55°C, 382 bp; IL-6: 56.1°C, 369 bp; PPAR-α: 55°C, 269 bp. The PCR products were visualized on 1.5% w/v agarose gel by staining with ethidium bromide.

Quantification of PCR products

To semiquantify the relative amounts of PPAR-α or IL-6 mRNA, the band intensities of amplified products were related densitometrically to the signal intensity of the respective glyceraldehyde-3-phosphate dehydrogenase PCR product of the same sample. Densitometrical evaluation was performed using a UVIDocMw software (Bio-Rad). Results from three different experiments were taken and expressed as percent of the control.

Western blot analysis

HaCaT keratinocytes were lysed in RIPA buffer (10 mM Tris pH 7.2, 150 mM NaCl, 0.1% SDS, 1% Triton, 1% deoxycholate, and 5 mM EDTA) supplemented with protease inhibitors (1 μM leupeptin, aprotinin, pepstatin A, and 1 mM PMSF). The protein content was determinated with the Bio-Rad protein assay and BSA for calibration. Twenty micrograms of total cell lysate proteins was resolved under reducing conditions using 8% SDS-polyacrylamide gel and transferred to nitrocellulose membrane (Amersharm Biosciences Co., Arlington Heights, IL). Protein transfer was checked with Ponceaus staining (Sigma), and after washing with PBS, the membrane was blocked with 5% non-fat dry milk in PBS with 0.05% Tween 20 for 1 hour at room temperature. After washing for 30 minutes in PBS and 0.05% Tween 20, the membrane was incubated with mAb against *PPARα* (clone 3B6, diluition 1:1,000) (Alexis Corporation, Switzerland) for 1 hour at room temperature. As a secondary antibody, goat anti-mouse IgG-HRP (diluition 1:1,000) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used, followed by ECL detection.

Statistical analysis

Values represent mean value ± SD. Statistical differences were evaluated using the Mann–Whitney test. Mean differences were considered to be significant when $P<0.05$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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