NOX2 Expression Is Increased in Keratinocytes After Burn Injury

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Reepithelialization is crucial for effective wound repair in burn wounds. Reactive oxygen species (ROS) have shown to be important in this. Recent studies suggest that NOX proteins produce ROS in keratinocytes. In the present study, we have studied NOX proteins in burn wounds, including the effect of C1-esterase inhibitor (C1inh) hereon, which is the endogenous inhibitor of complement activity whereof we have shown previously that it also increased the rate of reepithelialization in burn wounds. Skin tissue derived from healthy control Wistar rats (n = 6) were compared with burn-injured rats, with (n = 7) or without C1inh treatment (n = 7). After 14 days, rats were terminated. From the burn-injured rats, the entire wound and nonburned skin from the hind leg, that is, internal control was excised. From the control rats, dorsal skin was excised. In these skin samples, NOX2 and NOX4 were analyzed immunohistochemically. In nonburned rats, NOX2 was found in keratinocytes in both the basal layer and suprabasal layer of the epidermis; and the number of NOX2-positive keratinocytes was 367/mm² (254–378). In burned rats, the number of NOX2-positive keratinocytes was significantly increased in the newly forming epidermis in the burned area to 1019/mm² (649–1172), especially in the suprabasal layer, but significantly decreased in remote nonburned skin to 22/mm² (6–89). C1inh treatment counteracted these changes in epidermal NOX2 expression in burned rats, both in the burned area as in remote nonburned skin. No NOX4 expression was found in the epidermis in none of the groups. NOX2 expression was increased in keratinocytes in newly forming epidermis after burn injury. C1inh, a drug that increases the rate of reepithelialization, counteracted this effect. These results suggest a role for NOX2 in the reepithelialization of burn wounds.

Reepithelialization is an important process for effective wound repair in burn wounds.[1–3] It relies on the migration, proliferation, and differentiation of keratinocytes, the major cellular component of the epidermis.[4,5] Reactive oxygen species (ROS) are involved in these various cellular functions in keratinocytes.[6–10] Recent in vitro data, albeit not directly related to burn injury, suggest that keratinocytes produce ROS specifically via a family of specialized proteins, called the Nicotinamide Adenine Dinucleotide Phosphate (NADPH) Oxidases (NOX) proteins.[11–15] In humans, seven NOX proteins have been identified.[16] These NOX proteins have been shown to produce superoxide (O2–), the major source of ROS, and hydrogen peroxide (H2O2) and to play a role in several sometimes opposing cellular processes ranging from apoptosis to cell growth.[16–18] However, the role of NOX proteins in keratinocytes after burn has not been studied until now.

Nevertheless, several nonburn studies have described roles for NOX proteins in keratinocytes. Specifically, NOX2 and NOX4 have been reported to play a central role in the cell proliferation, migration, and host defense of human keratinocytes.[12,13] In addition, NOX proteins were also linked to keratinocyte apoptosis. Sun et al found in immortalized human skin keratinocytes (HaCaT) cells that apoptosis, induced by pro-apoptotic advanced oxidative protein products (AOPPs), coincided with increased cellular ROS levels and increased NOX4 expression.[15]

Moreover, Hara-Chikuma et al showed in primary keratinocyte cultures from mice with psoriasis, that ROS produced by NOX2 was required for NF-κB activation in keratinocytes in the development of psoriasis.[19]

Until now, only one study analyzed NOX proteins in burn wounds. Fang et al demonstrated in human skin tissue homogenates from burn wounds that NOX4 expression was significantly increased, however, they did not specify, which cells in the homogenate were positive for NOX4.[20] Neither did they analyze other NOX proteins herein.

We have previously shown that the complement inhibitor C1-esterase inhibitor (C1inh) significantly increased the rate of reepithelialization in a rat burn wound model.[21] C1inh is an endogenously expressed member of the serpin family of protease inhibitors, is among others present in blood plasma, and

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is a natural regulator of both the classical and lectin activation pathways of complement.[22] Interestingly, it was shown in renal ischemia/reperfusion models that C1inh inhibited NOX2 and NOX4 expression.[23] We therefore wondered whether C1inh would interfere with NOX expression in keratinocytes during burn injury.

As the role of NOX1 in keratinocytes is primarily linked to skin carcinogenesis,[24,25] we have focused on the putative role of NOX2 and NOX4 in burned skin, including the effect of C1inh hereon, in an in vivo rat burn wound model.

MATERIALS AND METHODS

All procedures were executed in agreement with the national guidelines and with permission of the Animal Experimental Committee of the VU University of Amsterdam.

Rat Burn Wound Model

We used skin tissue of a previous study in which we studied the role of C1inh treatment on local burn wound healing and systemic inflammation in a rat burn wound model.[21] In short, 12-week-old female Wistar rats ($n = 14$) were acclimatized for 1 week prior to the experiment. Fourteen rats were anesthetized using 2.5% isoflurane and received Temgesic (buprenorphine, 0.05 mg/kg, subcutaneous) as an analgesic. Then, a dorsal full-thickness burn wound (4 × 2 cm) was created on a shaved part of the skin by using a copper stamp (100 g) heated to 100°C for 15 seconds on the skin. Nonadhesive bandage was used to cover the wounds. Seven burn-injured rats received purified human plasma derived C1-esterase inhibitor (C1inh) (100 U/kg), (Sanquin, Amsterdam, The Netherlands) daily intravenously. The other seven burn-injured rats were not subjected to this treatment. After 14 days, all 14 rats were terminated. The complete wound as well as nonburned skin from the hind leg which served as internal control was excised. Six additional rats who did not receive a burn wound served as noninjured healthy controls. From these rats, dorsal skin was excised. The skin samples were immediately transferred to formalin for fixation and subsequently embedded in paraffin.

Immunohistochemistry

For immunohistochemical analysis, 4-µm sequential sections were dewaxed in xylene and rehydrated in ethanol (100%), followed by incubation in methanol/H_2O_2 0.3% solution for 30 minutes to block endogenous peroxidase activity. For antigen retrieval, slides were either boiled in a 10 mM citrate buffer (pH 6.0) (myeloperoxidase [MPO]) or 10 mM Tris/EDTA buffer (pH 9.0) (AE1/3, NOX2 NOX4 and Ki-67) in a microwave for 15 minutes.

Before the primary antibody, the sections to be stained with NOX2 and NOX4 were incubated with 1:50 normal rabbit serum (Dako, Glostrup, Denmark) for 10 minutes at room temperature. Then, the sections were incubated for 1 hour at room temperature with either 1:25 monoclonal mouse anti-human/rat NOX2 (Sanquin, Amsterdam, The Netherlands), or 1:25 monoclonal goat anti-human/rat NOX4 (Santa Cruz Biotechnology Inc, Heidelberg, Germany) or 1:100 monoclonal mouse anti-human/rat AE1/3 (Dako) to visualize keratinocytes, or 1:50 polyclonal rabbit anti-human/rat MPO (Dako) to visualize neutrophilic granulocytes, or 1:400 monoclonal rabbit anti-human/rat Ki-67 (clone SP6) (Thermoscientific, Fremont CA) to visualize proliferative cells. The sections were then incubated with either 1:25 polyclonal rabbit anti-mouse HRP (Dako) for NOX2, or 1:200 rabbit anti-goat HRP (Dako) for NOX4, or goat anti-mouse/rabbit envision HRP (Dako) for AE1/3 and MPO, or goat anti-rabbit envision HRP (Dako) for Ki-67 for 30 minutes at room temperature.

Staining was visualized by incubation with 3,3-diaminobenzidine (DAB, 0.1 mg/ml, 0.02% H_2O_2) (Dako) for 10 minutes at room temperature in the dark. Sections were then counterstained with hematoxylin, dehydrated, and covered. As a control, the same procedure was conducted, but instead of the primary antibody, normal antibody diluent (Immunologic, Duiven, The Netherlands) was used.

Immunohistochemical Analysis

In order to quantify the number of NOX2-positive keratinocytes per mm^2 surface area, the number of NOX2-positive keratinocytes per epidermis section was determined and the surface area of the epidermis was measured using QuickPHOTO Micro software windows version 3.1 (PROMICRA, Prague, Czech Republic). In burn wound areas, we analyzed the newly formed epidermis. As NOX2 is also expressed in neutrophils, sequential slides stained with the AE1/3 (keratinocyte marker) and MPO (neutrophilic granulocyte marker) to verify whether NOX2 staining was related with keratinocytes. NOX2 expression was compared between the basal layer of the epidermis versus the suprabasal layer.[1,2]

Statistical Analysis

GraphPad Prism 6 software (Graphpad Software Inc., California) was used for statistical analysis. Data were analyzed using Mann–Whitney *U* test or Wilcoxon test when appropriate. In order to control the type I error, a Bonferroni adjustment was applied to the resulting *P*-values. Corrected *P*-values ≤ .05 were considered significant. Results are described/depicted as: median ("line"); first quartile (Q1) – third quartile (Q3).

RESULTS

NOX Expression in Keratinocytes Is Increased in Newly Forming Epidermis After Burn Injury in Rats

NOX2 expression was found in epidermal keratinocytes in control rats and burned rats, both in the newly forming epidermis (Figure 1A) and in remote nonburned skin. NOX2 was also present in blood vessels in the dermis (data not shown). NOX2 was found especially in the nucleus of keratinocytes and to a lesser extent also in the cytoplasm. NOX2-positive keratinocytes were present in both the basal and suprabasal epidermal layers. Herein, there was no difference between the front of the neo-epidermis and the rest of the epidermis. To exclude that the presence of NOX2 in the epidermis was related to infiltrating neutrophilic granulocytes,[26] sequential slides were stained with keratinocyte marker AE1/3 (data not shown) and neutrophil marker MPO. No neutrophilic granulocytes were found in the epidermis (Figure 1A and B).

We subsequently quantified the number of NOX2-positive keratinocytes in the whole epidermis (Figure 2A). In control rats (367/mm^2 [254–378]), NOX2-positive keratinocytes

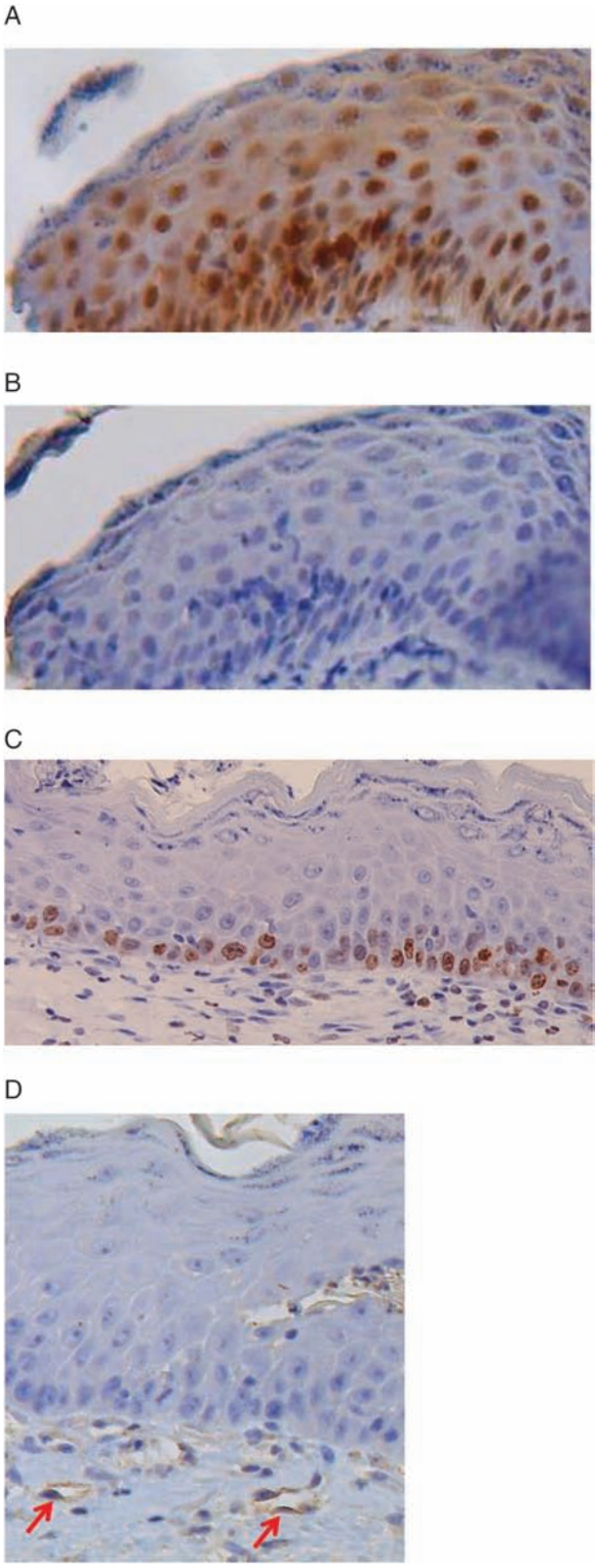

Figure 1. NOX2, MPO, Ki-67, and NOX4 staining in the burn wound. An example of NOX2 expression (A) and MPO staining (B) on serial slides in newly formed epidermis of the burn wound. (C) Ki-67 expression in keratinocytes in newly formed epidermis of the burn wound. (D) NOX4 expression in blood vessels (red arrows) in the dermis, at 14 days postburn.

were counted, expressed as median (Q1–Q3) (Figure 2A). In burned rats, in the newly forming epidermis, this number was significantly higher (1019/mm^2 [649–1172]), whereas in remote nonburned skin, this number was significantly lower (22/mm^2 [6–89]), compared to control rats (corrected $P \leq .01$ and $P \leq .05$, respectively).

Although NOX-produced ROS have been related to apoptosis induction, we did not find histological evidence for keratinocyte apoptosis (visualized as dyskeratotic cells[27]) in none of the groups.

As NOX proteins are also related to keratinocyte proliferation, we first verified whether burn injury had an effect on the number of proliferating cells in the epidermis, visualized via Ki-67 expression.[28] In control rats and burned rats (both in the newly forming epidermis and in remote nonburned skin), we found Ki-67 expression only in keratinocytes of the basal epidermal layer (Figure 1C). This indicates that as to be expected, proliferation was only found in the basal layer. No significant difference in the number of Ki-67-positive cells was found between the groups control, internal control and burned rats. The number of Ki-67-positive cells was 215/mm^2 (171–256), 7/mm^2 (1–31), and 164/mm^2 (86–322), respectively (Figure 2D).

Next, to analyze NOX2 expression in proliferating and/or differentiating keratinocytes, the number of NOX2-positive keratinocytes was quantified in the basal- versus the suprabasal epidermal layer.[1,2] In the basal layer of control rats the number of NOX2-positive keratinocytes was 261/mm^2 (159–327) (Figure 2B). In burned rats, the number of NOX2-positive keratinocytes in the newly forming epidermis (345/mm^2 [226–405]) was slightly higher than in control rats but not significantly. In contrast, in the remote nonburned skin, this number (20/mm^2 [4–84]) was significantly lower compared to both control rats and newly forming epidermis of burned rats (corrected $P \leq .05$). In the suprabasal layer of control rats, the number of NOX2-positive keratinocytes was 89/mm^2 (52–106) (Figure 2C). In burned rats, this number was significantly higher in the newly forming epidermis (668/mm^2 [319–845]; corrected $P \leq .01$) and significantly lower in the remote nonburned skin (2/mm^2 [1–21]; corrected $P \leq .05$).

Comparison between the basal- and suprabasal epidermal layers revealed that in control rats and the remote nonburned skin of burned rats, the number of NOX2-positive keratinocytes in the basal layer was higher than in the suprabasal layer ($P < .05$ and $P = .06$, respectively; Figure 2E). In contrast, in the newly forming epidermis of burned rats, the number of NOX2-positive keratinocytes in the basal layer was lower than in the suprabasal layer, albeit not significantly.

We also analyzed NOX4 expression in the skin. We however did not find NOX4 expression in keratinocytes in none of the groups, but only in blood vessels of the dermis (Figure 1D).

C1inh Treatment Normalizes NOX2 Expression in Keratinocytes in Burned Rats

We subsequently analyzed whether C1inh treatment affected NOX2 expression in keratinocytes in burned rats. In remote nonburned skin of C1inh-treated rats, the number of NOX2-positive keratinocytes of the whole epidermis (456/mm^2 [439–489]) was significantly increased compared to nontreated rats (22/mm^2 [6–89]; corrected $P \leq .01$) (Figure 3A). In contrast,

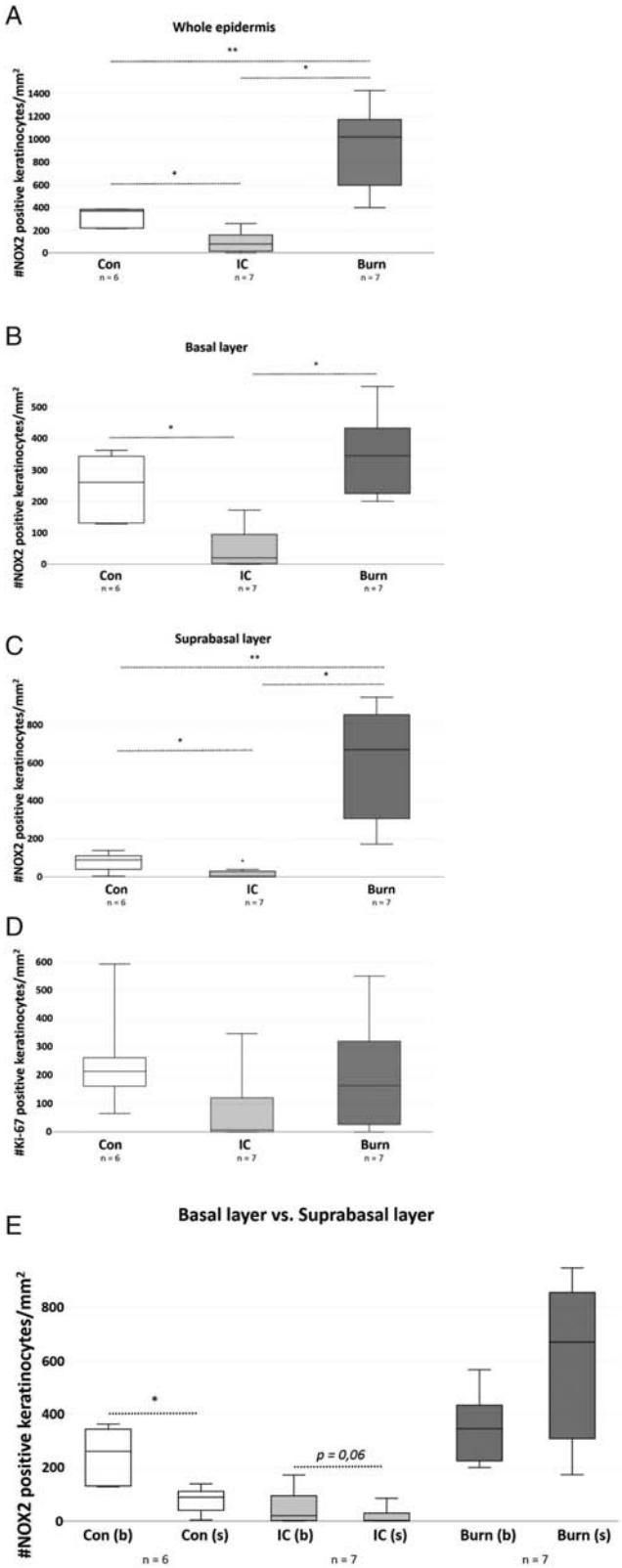

Figure 2. Quantification of NOX2 and Ki-67 expression in the skin. The number of NOX2 positive keratinocytes per mm2 in the whole epidermis (A), the basal layer (B), the suprabasal layers of the epidermis (C) and the number of Ki-67 positive keratinocytes per mm2 (D) in healthy skin from noninjured control rats (Con), burn-injured skin (Burn) and remote skin (Internal Control [IC]) from burn-injured rats, at 14 days postburn. (E) The number of NOX2 positive keratinocytes per mm2 in the basal layer (b) and the suprabasal layer (s) within control rats (Con), remote skin (internal control) from burn-injured rats (IC) and burned skin (Burn) at 14 days postburn. Median ("line"); first quartile (Q1) – third quartile (Q3). *= corrected $P \leq .05$.

the number of NOX2 positive keratinocytes in the newly forming epidermis was reduced by C1inh from 1019/mm² (649–1172) to 554/mm² (395–635) in burn-injured rats, although statistically not significant.

In the basal epidermal layer of remote nonburned skin, C1inh significantly increased the number of NOX2-positive keratinocytes to 250/mm² (221–280) compared to 20 mm² (4–84) in nontreated rats (corrected $P \leq .01$; Figure 3B). Whereas in the basal layer of newly forming epidermis, C1inh significantly reduced this number to 345/mm² (226–405) compared to 186/mm² (124–199) in nontreated rats (corrected $P \leq .01$). Also, in the suprabasal layer of remote nonburned skin, C1inh significantly increased the number of NOX2-positive keratinocytes to 174/mm² (132–215) compared to 2/mm² (1–21) in nontreated rats (corrected $P \leq .01$; Figure 3C). While in the newly forming epidermis, C1inh reduced this number to 357/mm² (254–459) compared to 668/mm² (319–845) in nontreated rats, although not significantly.

We subsequently analyzed whether C1inh treatment affected the number of Ki-67-positive keratinocytes in burned rats. However, no significant differences in the number of Ki-67-positive keratinocytes were found between C1inh-treated and nontreated rats: 300/mm² (197–340) and 164/mm² (86–322), respectively (Figure 3D). Both in nontreated and in C1inh-treated rats, Ki-67-positive keratinocytes were found only in the basal epidermal layer (data not shown).

DISCUSSION

Reepithelialization is crucial for effective wound healing after burn injury, in which keratinocytes play a pivotal role.[1–3] ROS have been demonstrated to be important for keratinocyte proliferation, migration, and apoptosis, processes central to reepithelialization. Previous in vitro data suggest that NOX proteins are an important source of ROS production in keratinocytes. Here, we show in burned rats that NOX2 expression was significantly increased in the newly formed epidermis while it was significantly decreased in remote nonburned skin compared to healthy control rats. This increased NOX2 expression in the newly formed epidermis was especially evident in the suprabasal layer. Interestingly, C1inh treatment, which we have shown before to increase the rate of reepithelialization,[29] normalized epidermal NOX2 expression in burned rats. Finally, NOX4 expression was only found in blood vessels of the dermis, not in the epidermis.

NOX proteins produce low levels of intracellular ROS that were found to serve as signaling intermediates. In this way, NOX2 as well as NOX4 are involved in various cellular signaling pathways including cell death, cellular differentiation, and proliferation.[31–33] Chamulitrat et al showed that NOX1, NOX2, and NOX4 mRNA were constitutively expressed in human immortalized keratinocytes (HaCaT cells) as detected

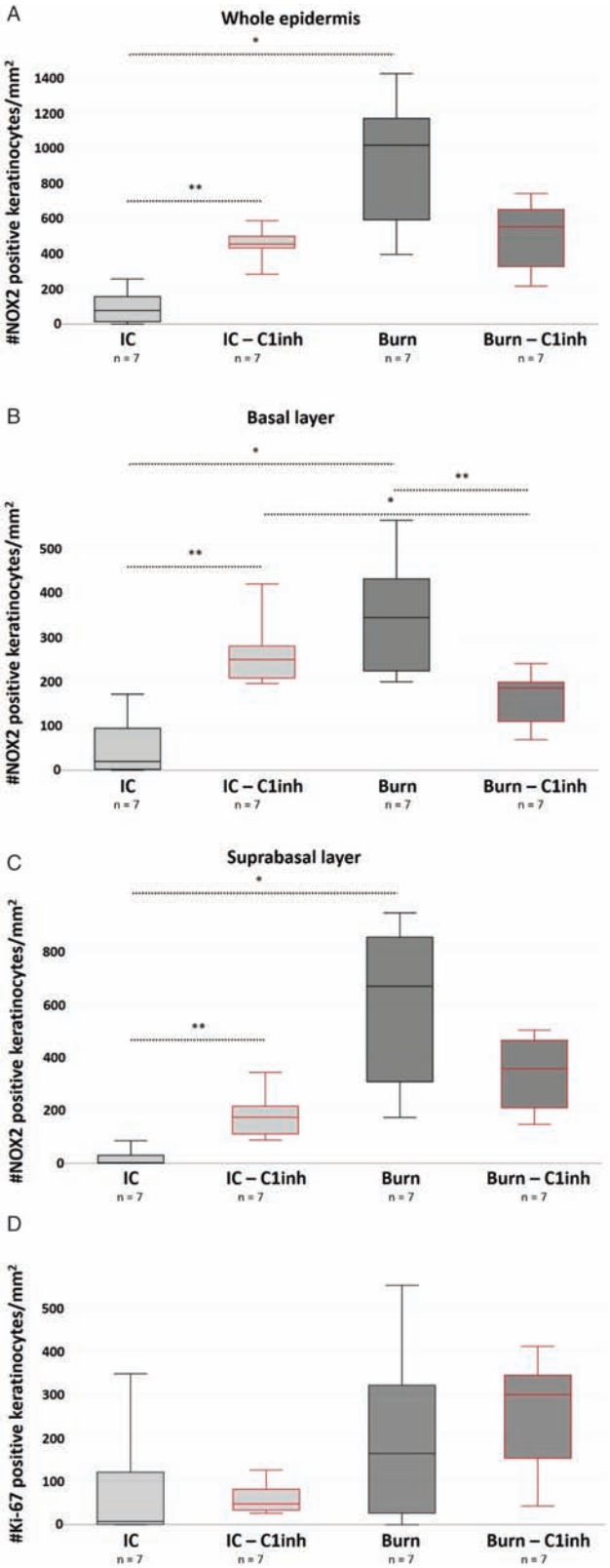

Figure 3. Effect of C1inh administration on NOX2 and Ki-67 expression in the skin. The number of NOX2-positive keratinocytes per mm2 in the whole epidermis (A), the basal layer (B), the suprabasal layer of the epidermis (C), and the number of Ki-67-positive keratinocytes per mm^2 (D) of remote skin (Internal Control [IC]) from burn-injured nontreated (IC) and burn-injured C1inh-treated (IC – C1inh) rats, and burned skin from burn-injured nontreated (Burn) and burn-injured C1inh-treated (Burn – C1inh) rats, at 14 days postburn. Median ("line"); first quartile (Q1) – third quartile (Q3). *= corrected $P \leq .05$. **= corrected $P \leq .01$. ***= corrected $P \leq .001$.

by RT-PCR.[12] However, NOX2 and NOX4 protein expression in keratinocytes was not yet demonstrated on protein level. In the present study, we presented for the first time that NOX2 is expressed in keratinocytes in both in healthy skin as well as in newly forming epidermis after burn injury, while we did not detect NOX4 in keratinocytes. The lack of NOX4 in keratinocytes in the rat is at odds with the NOX4 mRNA previously found in HaCaT cells.[12] Whether this is related to a difference in species or whether NOX4 is also absent from primary nonimmortalized human keratinocytes remains to be established.

In healthy skin from control rats and remote nonburned skin from burned rats, NOX2 was mainly expressed in keratinocytes in the basal epidermal layer. As confirmed with Ki-67 staining and as expected, virtually all keratinocytes in the basal layer are proliferating. This suggests that NOX2 may be involved in keratinocyte proliferation, also under physiological conditions. As NOX2-positive keratinocytes were also found in the basal layer of newly forming epidermis in burned rats, NOX2 may also play a role in keratinocyte proliferation under pathophysiological conditions. The results of a study by Hara-Chikuma et al who showed in primary keratinocyte cultures from mice with psoriasis that NOX2-produced ROS were required for keratinocyte proliferation,[19] support such a role.

The increase in NOX2 expression in the newly forming epidermis was especially evident in suprabasal keratinocytes. As suprabasal keratinocytes lose the ability to proliferate,[1,2] and we also did not observe any Ki-67-positive keratinocytes in this layer, it is unlikely that the increase in NOX2 expression in the suprabasal layer is associated with keratinocyte proliferation. Moreover, we did not find any dyskeratotic cells in the epidermis. Hence we cannot verify a role for NOX2 in keratinocyte apoptosis.

In the present study, we found that C1inh normalized NOX2 expression in keratinocytes both in the newly forming epidermis and in the remote nonburned skin of burned rats. Previously, we showed that treatment with C1inhsignificantly increased the rate of reepithelialization after burn injury in rats.[21] It is important to note that the reepithelialization of the burn wounds was ongoing both in C1inh-treated and nontreated rats, averaging 55 ± 4% and 38 ± 4% of the surface area of the burn wounds covered with newly formed epidermis, respectively.[21] This shows that the normalizing effects of C1inh on epidermal NOX2 expression were not the result of a completed reepithelialization. For the moment, it is unclear whether C1inh affects NOX2 expression in keratinocytes in a direct or an indirect manner, for instance, via an inhibitory effect on inflammation.

In conclusion, our results demonstrate that burn wounds have increased NOX2 expression in keratinocytes and that C1inh can avert this. However, this has to be studied further in order to explore the underlying mechanism in more detail.

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