Testosterone Stimulates Duox1 Activity through GPRC6A in Skin Keratinocytes*

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Background: The molecular mechanisms underlying the non-genomic activities of testosterone in keratinocytes are unknown.

Results: Testosterone stimulates Duox1 activity through GPRC6A leading to cell death in skin keratinocyte.

Conclusion: These results support an understanding of the molecular mechanism of testosterone-dependent apoptosis through Duox1-induced H2O2 generation.

Significance: These results provide a novel signaling cascade of testosterone-mediated redox regulation in keratinocytes.

Testosterone is an endocrine hormone with functions in reproductive organs, anabolic events, and skin homeostasis. We report here that GPRC6A serves as a sensor and mediator of the rapid action of testosterone in epidermal keratinocytes. The silencing of GPRC6A inhibited testosterone-induced intracellular calcium ([Ca2+]i) mobilization and H2O2 generation. These results indicated that a testosterone-GPRC6A complex is required for activation of Gαq protein, IP3 generation, and [Ca2+]i mobilization, leading to Duox1 activation. H2O2 generation by testosterone stimulated the apoptosis of keratinocytes through the activation of caspase-3. The application of testosterone into three-dimensional skin equivalents increased the apoptosis of keratinocytes between the granular and stratified corneum layers. These results support an understanding of the molecular mechanism of testosterone-dependent apoptosis in which testosterone stimulates H2O2 generation through the activation of Duox1.

Recently, reactive oxygen species (ROS) have been recognized as important messengers in cell signaling (1–3). Various agonists including growth factors, hormones, and neurotransmitters stimulate ROS generation to mediate physiological responses, such as cell proliferation, differentiation, and cell death in different cell types (1–5). Transient ROS generation by agonists may occur in the plasma membrane. It has been reported that NADPH oxidase (Nox) isoforms are primarily localized in the plasma membrane, and the activity of Nox appears to be regulated by the activation of various receptors (4). The Nox family includes seven isoforms (Nox1; gp91 phox, renamed Nox2; Nox3; Nox4; Nox5; Duox1; and Duox2) and is expressed in various tissues including colon, kidney, thyroid gland, testes, salivary glands, airways, and lymphoid organs, and it mediates ROS generation to coordinate tissue-specific functions. Duox1/2 isoforms differ from the other Nox homologs. They contain an additional peroxidase-like domain in the NH2-terminal region and intracellular Ca2+-binding EF-hand domain regions, which suggests that the activity of these isoforms is regulated by intracellular calcium mobilization.

The skin is the largest organ and protects the body. This protective barrier function largely relies on the stratum corneum, the end product of the terminal differentiation of epidermal keratinocytes (6). The stratum corneum is maintained through the continuous addition of new differentiating cornocytes surrounded by intercellular lipids; this tissue is constantly forming in the basal layer and being removed at the outermost surface. The balance between the differentiation and death of keratinocytes is crucial for the homeostasis of the epidermis (7). Many cytokines and growth factors stimulate permeability barrier formation, whereas testosterone seems to inhibit formation (8). Indeed, testosterone plays an important role in reproductive physiology and anabolic biological activities in multiple tissues and regulates the various functions of skin biology, such as sebaceous gland growth, differentiation, hair growth, skin barrier homeostasis, and wound healing (9–11).

Testosterone and dihydrotestosterone (DHT) activate the androgen receptor (AR) as nuclear receptor. The association of androgen with soluble AR results in its translocation to the nucleus and the stimulation of androgen-regulated gene

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expression. Recently, rapid non-genomic effects of testosterone have been reported in various cell types including smooth muscle cells, endothelial cells, Sertoli cells, prostate cells, and immune cells (12–15). However, the molecular mechanisms underlying the non-genomic activities of testosterone in keratinocytes are unknown.

Recently, emerging evidence has indicated that the orphan G protein-coupled receptor GPRC6A serves as a multiligand receptor for l-amino acids, calcium, osteocalcin, or testosterone and is expressed in various tissues, where it regulates many physiological processes (16–18). GPRC6A requires a positive allosteric modulator such as calcium for the rapid and transmembrane activity of testosterone. Indeed, a testosterone-GPRC6A complex mediates insulin secretion from β-cells, regulating glucose homeostasis, and confers a rapid response in prostate cells (19). Previous reports indicated that testosterone regulates ROS generation through the activation of Nox isozymes, suggesting a molecular connection between testosterone and cellular redox status (20). Although the Duox1 isozyme is expressed in epidermal keratinocytes, the molecular function of this isozyme is undefined. Here, we show that testosterone stimulates GPRC6A resulting in the sequential activation of a Gq-PLCβ-IP3-Ca2+ cascade in epidermal keratinocytes. Additionally, intracellular calcium mobilization mediated by testosterone induces the activation of Duox1 and the generation of H2O2, which leads to keratinocyte apoptosis. This report provides a molecular mechanism in which the non-genomic action of testosterone mediates the regulation of the cellular redox status.

**EXPERIMENTAL PROCEDURES**

**Chemical Reagents**—Testosterone (4-androsten-17β-ol-3-one, T1500), flutamide (4-nitro-3-trifluoromethylisobutyranilide, F9397), diphenylethionium chloride (DPI, D2926), BAPTA-AM (A1076), and xestospongin C (X2628) were purchased from Sigma. NPS2143 (SB262470) was purchased from Selleckchem. Fura-2-acetoxyethyl ester (Fura-2/AM, F-1201), pluronic-F127 (P3000MP), and 2',7'-dichlorofluorescin diacetate (DCF-DA, D-399) were obtained from Invitrogen. Peroxy-Orange 1 was obtained from Christopher J. Chang.

**Cell Culture**—Epidermal keratinocytes (MPEK-BL6) were purchased from CellnTec (Bern, Switzerland) and subcultured in keratinocyte growth medium (CellnTec). Cells were cultured in KBM medium (0.15 mM Ca2+) with keratinocyte growth medium growth supplements (Lonza, CC-3111).

**Quantitative RT-PCR**—The RNAs were extracted with 1 ml of TRIzol reagent (Molecular Research Center, Inc.) and 0.2 ml of chloroform and precipitated with 0.5 ml of isopropyl alcohol. The suspension was centrifuged at 12,000 × g at 4 °C for 15 min. One microgram of total RNA was reverse transcribed by the Reverse Transcription System (Promega, Madison, WI). Quantitative real-time PCR was performed using TaqMan® universal PCR master mix or SYBR® master mix. Pre-designed gene specific TaqMan probe and SYBR Green primers were purchased from Applied Biosystems. The temperature profile for the reaction was: 50 °C for 2 min, 95 °C for 20 s, and then 95 °C for 3 s and 60 °C for 30 s for 45 cycles. The relative quantity was obtained using the comparative threshold method and results were normalized against 18 S rRNA as an endogenous control.

**Gene Knockdown by siRNA Transfection**—For knockdown of Duox1, GPRC6A, and Gq, cells were transfected with control siRNA (ON-TARGETplus Non-targeting siRNA #2, D-001810-02-20), Duox1 (siGENOME Duox1 siRNA, D-047172-01-0005), GPRC6A (siGENOME Gprc6a siRNA- SMARTpool, M-054451-00-0005), mixtures of four highly functional, individual siRNAs), siRNA (Dharmacon), or Gq (FlexiTube GeneSolution for Gnaq SI02708713) siRNA (Qiagen) according to the manufacturer’s protocol.

**Immunoblotting**—Cells were chilled in lysis buffer (50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 0.1 mM 4-[(2-aminoethyl)benzenesulfonyl fluoride, 1 mM Na3VO4, 1 mM sodium fluoride, 1 μg/ml of aprotinin, 1 μg/ml of leupeptin, and 10% glycerol) for 30 min, then sonicated (at 10% amplitude for 1 s in a Branson sonifier equipped with a microtip), followed by a 30-min 14,000 × g centrifugation. After boiling in 5× SDS-PAGE sample buffer, the samples were subjected to 10% SDS-PAGE. The resolved proteins were electrotransferred to a nitrocellulose membrane. The membrane was immunoblotted with anti-Duox1 and GPRC6A (Santa Cruz Biotechnology), followed by horseradish peroxidase-conjugated goat anti-rabbit IgG antibody. Bands were visualized by chemiluminescence (Fujifilm LAS-3000).

**Measurement of Intracellular ROS**—After the confluent cells were stimulated, cells were washed with Hank’s balanced salt solution and incubated for 10 min in the dark at 37 °C with the same solution containing 10 μM DCF-DA (Molecular Probes). The cells were then examined with a laser-scanning confocal microscope (model LSM 510, Carl Zeiss) equipped with an argon laser tuned to an excitation wavelength of 488 nm, an LP505 emission filter (515 to 540 nm), and a Zeiss Axiovert 100× objective lens. Images were digitized and stored at a resolution of 512 by 512 pixels. Five groups of cells were randomly selected from each sample, and the mean relative fluorescence intensity for each group of cells was measured with a Zeiss vision system (LSM510, version 2.3) and then averaged for all groups. All experiments were repeated at least three times.

**Measurement of Intracellular H2O2 by Peroxy-Orange 1**—After the cell culture media was swapped out, cells were incubated for 15 min in the dark at 37 °C with 5 μM PO-1 in Dulbecco’s PBS, and then stimulants were added to the dye/Dulbecco’s PBS cell mixture. The cells were then examined with a laser scanning confocal microscope (model LSM 510, Carl Zeiss) equipped with an argon laser tuned to an excitation wavelength of 488 nm. Five groups of cells were randomly selected from each sample, and the mean relative fluorescence intensity for each group of cells was measured with a Zeiss vision system (LSM510, version 2.3) and then averaged for all groups. All experiments were repeated at least three times.

**Measurement of Intracellular Calcium**—Keratinocytes (106 cells) were seeded onto 22-mm glass coverslips and cultured for 24 h, and then incubated for 16 h in serum-free medium. Cells were loaded with 5 μM Fura-2 AM and 0.05% pluronic acid for 1 h at room temperature in HEPES buffer (140 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM glucose, 10 mM HEPES, adjusted to pH 7.4 with HCl). Cells were then washed twice with...
HEPES buffer. The coverslips were next attached to a chamber slide mounted onto the microscope. HEPES buffer (140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM glucose, 1 mM EDTA, 1 mM EGTA, 10 mM HEPES, adjusted to pH 7.4 with HCl) was perfused at slow speed through the chamber with 100 nm testosterone applied locally to cells by pressure ejection via a micropipette. Calcium measurements were obtained by direct imaging via a real time fluorescent confocal imaging system. Images were acquired at 340/380 nm for excitation and 510 nm for emission. Fura 2 fluorescence was calibrated according to the method described by Grynkiewicz et al. (21). For this purpose, the cells were exposed to 5 μM ionomycin in modified HEPES solution containing either 3 mM Ca²⁺ or 5 mM EGTA to obtain the maximum (R_max) and minimum (R_min) ratio of fluorescence (R), respectively. [Ca²⁺], was calculated according to the equation [Ca²⁺] = Kd × (R - R_min)/(R_max - R), with use of the dissociation constant (Kd) of Fura 2, where β is the ratio of the 380-nm excitation signals of ionomycin-treated cells at 5 mM EGTA and 3 mM Ca²⁺. Experiments were repeated at least three times with similar results.

Measurement of IP₁—IP₁ ELISA (721P1PEA, Cisbio) was used according to the manufacturer’s instructions to measure the IP₁ produced in the cell after G protein-coupled receptor activation. Intracellular IP₁, a measure of the degradation products of InsP₃ and a surrogate for InsP₃ levels, was measured after LiCl (50 mM) treatment to prevent the degradation of IP₁ into myo-inositol. Briefly, 5 × 10⁵ cells were plated in a 24-well culture plate and incubated overnight, and then incubated for 16 h in serum-free medium. Cells were stimulated with testosterone for the indicated times and lysed for 30 min. The lysates were then sonicated and centrifuged. After transfer of the supernatant into the ELISA plate supplied with the kit, samples were incubated for 3 h with IP₁-HRP conjugate and anti-IP₁ mAb. After a washing step, the revelation is carried out by addition of the HRP substrate 3,3′-diaminobenzidine. The reaction was stopped and the optical density (OD) was read at 450 nm with an optional correction between 610 and 650 nm (correction for optical imperfections in the plate). Experiments were repeated at least three times with similar results.

Mitochondrial Membrane Potential—The measurement of mitochondrial transmembrane potential was performed using the JC-1-based assay (Invitrogen). For each sample, cells were suspended in 1 ml of warm medium at ~1 × 10⁶ cells/ml. Then 1 μl of 5 mM JC-1 (5 μM final concentration, Molecular Probes) was added and the cells were incubated at 37 °C, in 5% CO₂, for 15 to 30 min. The cells were then transferred to an agarose-containing 24-well plate and moved to a new medium-containing dish for subsequent testosterone (Sigma) treatment every 24 h for 48 h. The skin equivalent (SEs), Epiderm FT-200 (MatTek Corp.), were removed from agarose-containing 24-well plates and moved to 6-well plates filled with Epiderm Full Thickness medium. Epiderm FT-200 SEs were cultured at 37 °C, in 5% CO₂ for 1 day and then transferred to a new medium-containing dish for subsequent testosterone (Sigma) treatment every 24 h for 48 h.

Immunostaining—SEs were cryopreserved in frozen section compound (FSC 22®, Leica Microsystems), sectioned at 6 μm, and stained with hematoxylin and eosin for standard evaluation. To compare the number of apoptotic cells, replicate cryosections were incubated with the In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics) for 40 min at room temperature. Nuclei were counterstained with Fluorescent Mounting Medium with 4,6-diamidino-2-phenylindole (E19-18, Golden Bridge International, Inc., Mukilteo, WA) for 5 min. The reactivity was evaluated under a microscope (Bx-41, Olympus, Japan) equipped with reflected fluorescence system, and photomicrographs were taken using a microscope digital camera (DP72, Olympus, Japan).

Statistic Analysis—All data are presented as mean ± S.E. Significant differences between treatment groups were identified with a t test. p Values less than 0.05 were considered statistically significant.
**RESULTS**

Connection between the Non-genomic Activity of Testosterone and ROS Generation in Epidermal Keratinocytes—The rapid, non-genomic activity of testosterone has been documented in various tissues. Specifically, testosterone induces the mobilization of intracellular calcium ([Ca\(^{2+}\)]\(_i\)) within a few minutes in Sertoli cells, prostate cells, and smooth muscle cells, which indicates that the rapid response to testosterone is mediated by an AR-independent cascade (12, 13).

To evaluate the non-genomic activity of testosterone in epidermal keratinocytes, we first measured [Ca\(^{2+}\)]\(_i\), mobilization as a hallmark of the non-genomic action of testosterone. The stimulation of epidermal keratinocytes with testosterone resulted in rapid and transient [Ca\(^{2+}\)]\(_i\) mobilization within 1 min (Fig. 1A). To test whether testosterone stimulates ROS generation in keratinocytes, we measured the intracellular levels of ROS by measuring the fluorescence of 2',7'-DCF-DA using a confocal microscope (22). The stimulation of epidermal keratinocytes with testosterone revealed significantly increased ROS generation within 5 min, which then decreased after 30 min (Fig. 1, B and C). It well known that physiological testosterone concentrations are 15–30 nM (23). We tested the effect of various concentrations of testosterone on ROS generation in epidermal keratinocytes. Testosterone stimulated the ROS production of epidermal keratinocytes in a concentration-dependent manner (Fig. 1D).

To identify the ROS species in the cells, we used PO-1 dye, which is specifically sensitive to H\(_2\)O\(_2\) (24). Increasing PO-1 fluorescence, indicating H\(_2\)O\(_2\) generation, was detected in epidermal keratinocytes in response to testosterone (Fig. 1E).

Therefore, the predominant species of ROS induced by testosterone in epidermal keratinocytes is H\(_2\)O\(_2\).

To explore whether testosterone-mediated H\(_2\)O\(_2\) generation is dependent on non-genomic activity, we used flutamide as an AR antagonist (25). The pre-treatment of epidermal keratinocytes with flutamide failed to inhibit testosterone-induced H\(_2\)O\(_2\) (Fig. 1F). These results indicate that the H\(_2\)O\(_2\) generation is independent from AR activation.

Testosterone-mediated [Ca\(^{2+}\)]\(_i\) Mobilization Induces the Activation of Duox1 in Keratinocytes—We explored whether testosterone-mediated H\(_2\)O\(_2\) generation is regulated by Nox activity. The pretreatment of the keratinocytes with DPI as a Nox inhibitor resulted in a significant reduction of H\(_2\)O\(_2\) generation in response to testosterone (Fig. 2A). The result indicated that testosterone-mediated H\(_2\)O\(_2\) generation might be from enhancing Nox activity instead of an AR-dependent pathway. We next tested the expression levels of Nox isozymes in epidermal keratinocytes. Quantitative real-time PCR indicated that Duox1 was the predominant Nox isozyme in epidermal keratinocytes; other Nox isozymes were barely detectable (Fig. 2B). This result indicated that the Duox1 isozyme may be responsible for testosterone-induced ROS generation in epidermal keratinocytes.

To explore the function of Duox1 in testosterone-induced H\(_2\)O\(_2\) generation, Duox1-targeting siRNA was transfected into keratinocytes and then H\(_2\)O\(_2\) generation was measured with DCF-DA fluorescence. The stimulation of Duox1-silenced keratinocytes with testosterone failed to generate H\(_2\)O\(_2\),
Testosterone-dependent Duox1 Activation

Figure 2. Testosterone (T)-mediated intracellular calcium mobilization induces the activation of Duox1 in keratinocytes. A, effects of a Nox inhibitor on testosterone-induced ROS generation. Cells were pretreated with the Nox inhibitor, DPI (10 μM), for 30 min and ROS generation was monitored after 5 min of testosterone treatment. Data are presented as mean ± S.E. (n = 5). B, expression of Nox isoforms in keratinocytes measured by real-time PCR analysis with primers specific for each Nox isoform and 18S rRNA as the reference gene. C, testosterone stimulates H2O2 generation by Duox1 in keratinocytes. Cells were transfected with either Duox1 siRNA or control siRNA with RNAiMAX. After 100 nM testosterone treatment, the generation of H2O2 was monitored by confocal microscopic analysis of DCF fluorescence. Data are presented as mean ± S.E. (n = 3). D, the protein level of Duox1 was analyzed by immunoblot. E, effects of intracellular Ca2+ chelation on testosterone-induced ROS generation in keratinocytes. Cells were pretreated with the intracellular Ca2+ chelator, BAPTA/AM (10 μM), for 30 min, and ROS generation was monitored after 5 min of testosterone treatment by laser-scanning confocal microscopy. Data are presented as mean ± S.E. (n = 5).

We evaluated the effect of GPRC6A-mediated [Ca2+]i mobilization on the activation of Duox containing a calcium binding domain in the NH2-terminal region. The silencing of GPRC6A by transfection of GPRC6A siRNA resulted in decreased H2O2 generation in response to testosterone, suggesting that GPRC6A-dependent [Ca2+]i mobilization stimulates the activity of Duox1 (Fig. 3C). Moreover, the pretreatment of epidermal keratinocytes with NPS2143, a non-competitive antagonist of GPRC6A (27, 28), resulted in significantly reduced testosterone-induced H2O2 generation (Fig. 3E). These results indicate that GPRC6A plays an important role in testosterone-dependent H2O2 generation in epidermal keratinocytes.

Effect of Downstream Signaling Networks of GPRC6A on H2O2 Generation in Keratinocytes—GPRC6A is known to be coupled with the Gq protein leading to IP3 generation and intracellular calcium mobilization (29). We tested the effect of Gq on H2O2 generation by the stimulation of testosterone. Transfection of epidermal keratinocytes with Gq-targeted siRNA resulted in decreased H2O2 generation compared with control siRNA in response to testosterone (Fig. 4, A and B). Next we measured testosterone-induced IP3 generation. Gq-dependent PLCβ stimulates the hydrolysis of phosphatidylinositol 4,5-bisphosphate into diacylglycerol, which activates PKC and IP3, which stimulates [Ca2+]i mobilization through binding to the IP3 receptor (IP3R) in the endoplasmic reticulum (30). The metabolism of IP3 by phosphatase is too fast to detect in cells. Here, we measured inositol monophosphate (IP1) as the end product of IP3 in epidermal keratinocytes (31). The stimulation...
of epidermal keratinocytes with testosterone resulted in rapid IP₃ production (Fig. 4).

Xestospongin C has been reported as a membrane-permeable inhibitor of the IP₃-mediated [Ca²⁺/H]ᵢ mobilization by blocking the IP₃ receptor (32). However, 20 µM xestospongin C has no effect on ryanodine receptor-mediated Ca²⁺ release (33). We evaluated the function of xestospongin C in testosterone-dependent H₂O₂ generation. The pretreatment of epidermal keratinocytes with xestospongin C prevented the generation of H₂O₂ in response to testosterone (Fig. 4). These results indicated that the GPRC6A-Gq-IP₃-[Ca²⁺/H]ᵢ signaling cascade induced by testosterone is essential for the Duox1 activation leading to H₂O₂ generation in epidermal keratinocytes.

Testosterone-dependent H₂O₂ Stimulates the Apoptosis of Epidermal Keratinocytes—Several lines of evidence indicated that testosterone induces the apoptosis of various cell types including smooth muscle cells, endothelial cells, neuronal cells, renal cells, and dermal papilla cells (34–38). Here, we showed that testosterone induces their signaling network resulting in H₂O₂ generation in epidermal keratinocytes. Therefore, we hypothesized that testosterone-dependent H₂O₂ generation is involved in the apoptosis of keratinocytes. It has been well established that disruption of mitochondrial integrity was affected in the early apoptotic process (39). Therefore, we measured depolarization of the outer mitochondrial membrane as a marker of apoptosis in response to testosterone in keratinocytes. Stimulation of keratinocytes with testosterone resulted in decreased mitochondrial membrane potential (Fig. 5A). To determine whether testosterone induced keratinocyte apoptosis, we performed the TUNEL assay. The stimulation of epidermal keratinocytes with testosterone resulted in significantly increased TUNEL labeling compared with unstimulated cells (Fig. 5B).

The pretreatment of keratinocytes with DPI (Nox inhibitor) or BAPTA-AM ([Ca²⁺/H]ᵢ chelator) resulted in a significant inhibition of testosterone-induced apoptosis (Fig. 5C). To evaluate the function of Duox1 or GPRC6A in testosterone-dependent apoptosis, knockdown of Duox1 or GPRC6A was performed. Interestingly, silencing Duox1 or GPRC6A failed to induce testosterone-dependent apoptosis in epidermal keratinocytes (Fig. 5D). Furthermore, pretreatment of keratinocytes with NPS2143, an inhibitor of GPRC6A, completely blocked testosterone-induced apoptosis (data not shown). Because activation of caspase-3 is a hallmark of apoptosis, we measured caspase-3 activity in response to testosterone (34, 36, 40). The incubation of epidermal keratinocytes with testosterone revealed increasing caspase-3 activity in response to testosterone (Fig. 5E). To examine whether activation of caspase-3 is induced by GPRC6A and Duox1 activity, we tested the effect of knocking down GPRC6A or Duox1 on the activation of caspase-3. The transfection of epidermal keratinocytes with GPRC6A siRNA or Duox1 siRNA prevented caspase-3 activation (Fig. 5F).
To confirm the testosterone-induced apoptosis of keratinocytes, we examined the effects of testosterone on three-dimensional in vitro SEs models. Treatment of SEs with testosterone increased TUNEL-positive keratinocytes between the granular and stratified corneum layers (Fig. 6A, right lower panel). The number of apoptotic cells with flattened nuclei was increased in the same region based on H&E staining of testosterone-treated SEs (Fig. 6A, left lower panel). An immature cornified layer was increased in testosterone-treated SEs compared with control SEs (Fig. 6A, left lower panel). Statistical analysis revealed that testosterone-treated SEs had 3-fold more TUNEL-positive cells than control SEs (Fig. 6B).

DISCUSSION

It is well established that testosterone is converted to DHT by 5α-reductase and that DHT then binds to the androgen receptor. This complex translocates into the nucleus, which leads to activating the expression of various genes. The genomic activity of testosterone regulates the development of reproductive organs and stimulates muscle and bone formation. Although the expression of the androgen receptor in keratinocytes is low (41), testosterone is involved in epidermal permeability barrier formation and wound healing, which indicates that testosterone regulates skin homeostasis through an AR-independent pathway (Fig. 5C).

Because intracellular calcium mobilization within minutes is a hallmark of the non-genomic activity of testosterone in many cell types (12–15), we first revealed that the orphan G protein-coupled receptor GPRC6A mediates the activity of testosterone in epidermal keratinocytes (Fig. 3). Our interest in this report focused on the molecular connection between the rapid, non-classical activity of testosterone and regulation of the cellular redox state. Because the Duox1 isozyme, the major isoform of Nox in epidermal keratinocytes, contains an EF-hand calcium-binding site, the connection of Duox1 isozyme activation with [Ca^{2+}]i mobilization by testosterone was explored. Silencing either GPRC6A or Gq in epidermal keratinocytes completely inhibited testosterone-mediated H2O2 generation (Figs. 3C and 4A). Moreover, pretreatment of epidermal keratinocytes with BAPTA, an intracellular Ca^{2+} scavenger, resulted in suppressed H2O2 generation in response to testosterone (Fig. 2E). Thus, our results indicated that the testosterone-GPRC6A-mediated signaling cascade, including rapid [Ca^{2+}]i release, resulted in increased H2O2 generation through Duox1 activation (Fig. 7).

Several lines of evidence indicate that various agonists stimulate intracellular ROS as second messengers in cells. It has been shown that Nox/Duox isozymes in the plasma membrane induce intracellular ROS generation. Nox and Duox isozymes...
share highly similarity. Their NĤ₃-terminal regions contain a heme group and their COOH-terminal regions bear NADPH- and FAD-binding sites. Transferring electrons from NADPH to O₂ through FAD and heme groups leads to ROS generation. Based on the structure of the Nox/Duox isozymes, the direction of ROS generation is inside to out. Recently, growth factors and

FIGURE 5. Testosterone-mediated H₂O₂ stimulates apoptosis of epidermal keratinocytes. A, depolarization of mitochondrial membrane potential. Keratinocytes were incubated with or without testosterone (100 nM) for 24 or 36 h, and then fixed, washed in PBS, and stained with Tdt-UDP nick end labeling for 1 h at 37 °C. Apoptotic cells were visualized by fluorescence microscopy. B, cells were pretreated with flutamide (100 nM) for 2 h, DPI (10 μM) for 30 min, and BAPTA/AM (10 μM) for 30 min and incubated for 24 h in the absence or presence of testosterone. Samples were stained with TUNEL and analyzed. The percentage of fluorescent cells in 10 random high-power fields was determined. Data were analyzed by Student’s t test. C, the percentage of fluorescent cells in 10 random high-power fields was determined. Data were analyzed by Student’s t test. E, caspase-3 activity assay. Testosterone (100 nM) was added to serum-free keratinocyte basal medium (KBM, Lonza) for 24 h. Lysates were assayed for caspase-3 activity using a caspase-3 fluorometric assay kit (Calbiochem). F, to evaluate the role of Duox1 and GPRC6A on DNA damage, siRNA was used against Duox1 and GPRC6A. Duox1 and GPRC6A siRNA-treated and untreated keratinocytes were incubated for 24 h in the absence or presence of testosterone. Samples were stained with TUNEL and analyzed. B-D, the percentage of fluorescent cells in 10 random high-power fields was determined. Data were analyzed by Student’s t test. *, comparison with testosterone-treated control group, p < 0.0000005; **, p < 0.000005. E, caspase-3 activity assay. Testosterone (100 nM) was added to serum-free keratinocyte basal medium (KBM, Lonza) for 24 h. Lysates were assayed for caspase-3 activity using a caspase-3 fluorometric assay kit (Calbiochem). F, to evaluate the role of Duox1 and GPRC6A on caspase-3 activity, siRNA was used against Duox1 and GPRC6A. Duox1 and GPRC6A siRNA-treated and untreated keratinocytes were incubated for 24 h in the absence or presence of testosterone. Lysates were assayed for caspase-3 activity. E and F, data are presented as mean ± S.E. (n = 3). Silences of Duox1 and GPRC6A are as described in the legends to Figs. 2D and 3D.

FIGURE 6. Effect of testosterone (T) on apoptosis of skin equivalents and keratinocytes in C57BL6. Skin equivalents were treated with testosterone (10 μM) every 24 h for 48 h. A, SEs were stained with H&E (left panel) or TUNEL (right panel). The upper panel shows an untreated S.E. and the lower panel shows a testosterone-treated SE. Many TUNEL-positive cells are observed only in the testosterone-treated SE. Arrows indicate the cells showing the characteristic morphology of apoptosis in H&E staining. Scale bar = 50 μm (SC, stratum corneum; SG, stratum granulosum; SS, stratum spinosum; SB, stratum basale; D, dermis). B, TUNEL-positive cells are counted in SEs treated with or without testosterone. The data shown are the mean ± S.E. of the results from 4 independent sample preparations; p < 0.01.
cytokines were shown to stimulate intracellular ROS generation through the formation of redox-active endosomes (42, 43). IL-1 binding to IL-1 receptor induces endosome formation including a Nox2 complex for the generation of intracellular ROS generation, which results in the activation of a cell signaling network. In this report, we did not provide information that testosterone stimulates endosome formation including the Duox1 isozyme. However, it is known that the COOH-terminal region of G protein-coupled receptors bears machinery to induce endocytosis (44). Therefore, it is likely that the testosterone-GPRC6A-Duox1 complex stimulates redox-active endosome formation, which results in intracellular H$_2$O$_2$ production in epidermal keratinocytes.

It has been reported that receptor-mediated cell signaling triggers ROS generation for cell growth and differentiation through activation of Nox isoforms (45). Nox-mediated ROS generation functions as second messenger in cell signaling and influences the activity of various downstream cytosolic proteins. For example, protein-tyrosine phosphatases are well known downstream targets of Nox-mediated ROS generation. Specifically, the sulfhydryl group of cysteine residues in the active center is oxidized to sulfenic acid by ROS converting the enzyme to an inactive form that in turn promotes tyrosine phosphorylation of cytosolic proteins (46, 47). Another example is the activation of apoptosis signal-regulating kinase-1 (ASK-1) by ROS. It is well known that ASK-1 interacts with thioredoxin as a repressor. Nox-mediated ROS by various stresses oxidize thioredoxin allowing dissociation of ASK-1, which mediates cell death (47, 48). In these events, oxidation of protein-tyrosine phosphatase and dissociation of ASK-1 indeed takes place within a short time span (minutes) but activation of their downstream signaling cascades, disruption of mitochondrial integrity, and gene expression changes require longer (hours).

It has been generally accepted that the generation of ROS by exogenous and endogenous stimuli plays an important role in the homeostasis of skin (49, 50). Specifically, the long-term effect of oxidative stress induced by exogenous stimuli such as UV is implicated in skin aging. UV-induced ROS generation is known to create DNA adduct and accelerate the aging process (49). Moreover, the topical application of linolein hydroperoxide stimulates the apoptosis of keratinocytes by inducing apoptosis-related genes (51). Even if agonist-dependent H$_2$O$_2$ generation acts as a second messenger in cell signaling, uncontrolled ROS generation induces apoptosis through the activation of caspase, expression of the Bcl2 family, or dysfunction of mitochondria (52). These previous reports indicated that ROS-induced apoptosis is related to skin cell aging. According to the notion that oxidative stress induces skin cell aging, the signaling cascade induced by testosterone stimulates Duox1-mediated H$_2$O$_2$ generation, which results in the apoptosis of keratinocytes. In testosterone-treated SE models, TUNEL-positive cells were significantly increased between the granular and stratified corneum layers, which indicates that testosterone regulates homeostasis of keratinocytes through the generation of H$_2$O$_2$ (Fig. 6). It has been reported that testosterone can interfere with the epidermal permeability barrier. Moreover, a replacement for the testosterone-castrated group showed a functional alteration of the epidermal barrier, including the formation of an abnormal cornified envelope leading to hyperkeratosis. Increased DHT has been reported to play a role in follicular hyperkeratinization in acne vulgaris (53). Increased caspase-3 is also known to be involved in the pathogenesis of various skin diseases, such as lichen planus (54). Taken together, these previous reports and our results provide a new concept for understanding the homeostasis of keratinocytes.

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REFERENCES

1. Block, K., and Gorin, Y. (2012) Aiding and abetting roles of NOX oxidases in cellular transformation. Nat. Rev. Cancer 12, 627–637
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2. Lassègue, B., San Martín, A., and Griendling, K. K. (2012) Biochemistry, physiology, and pathophysiology of NADPH oxidases in the cardiovascular system. *Circ. Res.* **110**, 1364–1390

3. Bae, Y. S., Oh, H., Rhee, S. G., and Yoo, Y. D. (2011) Regulation of reactive oxygen species generation in cell signaling. *Mol. Cells* **32**, 491–509

4. Petry, A., Weiitnauer, M., and Görlach, A. (2010) Receptor activation of NADPH oxidases. *Antioxid. Redox. Signal.* **13**, 467–487

5. Aguirre, J., and Lambeth, J. D. (2010) Nox enzymes from fungus to fly to mammals. *Free Radic. Biol. Med.* **49**, 1342–1353

6. Elias, P. M. (1983) Epidermal lipids, barrier function, and desquamation. *J. Invest. Dermatol.* **80**, 44–49

7. Candi, E., San Martín, A., and Griendling, K. K. (2012) Biochemistry, physiology, and pathophysiology of NADPH oxidases in the cardiovascular system. *Circ. Res.* **110**, 1364–1390

8. Feingold, K. R., and Denda, M. (2012) Regulation of permeability barrier homeostasis. *Clin. Dermatol.* **30**, 263–268

9. Zouboulis, C. C., and Degitz, K. (2004) Androgen action on human skin: from basic research to clinical significance. *Exp. Dermatol.* **13**, 5–10

10. De Gendt, K., and Verhoeven, G. (2012) Tissue- and cell-specific functions of the androgen receptor revealed through conditional knockout models in mice. *Mol. Cell. Endocrinol.* **352**, 13–25

11. Zouboulis, C. C., Chen, W. C., Thornton, M. J., Qin, K., and Rosenfield, R. (2007) Sexual hormones in human skin. *Horm. Metab. Res.* **39**, 85–95

12. Loss, E. S., Jacobus, A. P., and Wassermann, G. F. (2011) Rapid signaling responses in Sertoli cell membranes induced by follicle stimulating hormone and testosterone: calcium influx and electrophysiological changes. *Life Sci.* **89**, 577–583

13. Foradori, C. D., Weiser, M. J., and Handa, R. J. (2008) Non-genomic actions of androgens. *Front. Neuroendocrinol.* **29**, 169–181

14. Rahman, F., and Christian, H. C. (2007) Non-classical actions of androgens. *Trends Endocrinol. Metab.* **18**, 371–378

15. Simoncini, T., and Genazzani, A. R. (2003) Non-genomic actions of sex hormones. *Experientia* **59**, 281–292

16. Pi, M., and Quarles, L. D. (2012) Multiligand specificity and wide tissue distributions of androgens and gonadotropins. *Clin. Chem.* **49**, 1381–1395

17. Pi, M., Chen, L., Huang, M. Z., Zhu, W., Ringhofer, B., Luo, J., Christenson, W. F., and Zumoff, B. (1977) The effect of flutamide on testosterone metabolism and the plasma levels of androgens and gonadotropins. *J. Clin. Endocrinol. Metab.* **45**, 1224–1229

18. Pi, M., Chen, L., Huang, M. Z., Zhu, W., Ringhofer, B., Luo, J., Christenson, W. F., and Zumoff, B. (1977) The effect of flutamide on testosterone metabolism and the plasma levels of androgens and gonadotropins. *J. Clin. Endocrinol. Metab.* **45**, 1224–1229

19. Pi, M., Faber, P., Ekema, G., Jackson, P. D., Ting, A., Wang, N., Fontilla-Peule, M., Mays, R. W., Brunden, K. R., Harrington, J. J., and Quarles, L. D. (2008) Identification of a novel extracellular cation-sensing G-protein-coupled receptor. *J. Biol. Chem.* **283**, 40201–40209

20. Berridge, M. J. (2009) Inositol trisphosphate and calcium signalling mechanisms. *Biochim. Biophys. Acta* **1793**, 933–940

21. Blattermann, S., Peters, L., Ottersbach, P. A., Bock, A., Konya, V., Weaver, C. D., Gonzalez, A., Schröder, R., Tyagi, R., Luschign, P., Gáb, J., Hennen, S., Ulven, T., Pardo, L., Mohr, K., Gutschow, M., Heinemann, A., and Kostenis, E. (2012) A biased ligand for OXETR-un couples Go and Gβγ signaling within a heterotrimer. *Nat. Chem. Biol.* **8**, 631–638

22. Gref, R., Shawl, A. I., Kim, S. H., and Kim, U. H. (2012) Cooperative interaction between reactive oxygen species and Ca2+ signals contributes to angiotensin II-induced hypertrophy in adult rat cardiomyocytes. *Am. J. Physiol. Heart Circ. Physiol.* **302**, H901–H909

23. Gafni, J., Gafni, J., and Malhotra, S. (1994) Non-classical actions of testosterone: an update. *Trends Endocrinol. Metab.* **17**, 302–306

24. Bowles, D. K., Maddali, K. K., Dhulipala, V., and Koricz, D. H. (2007) PKCδ mediates anti-proliferative, pro-apoptotic effects of testosterone on coronary smooth muscle. *Am. J. Physiol. Cell Physiol.* **293**, C805–C813

25. Ling, S., Dai, A., Williams, M. R., Myles, K., Dilley, R. J., Komesaroiff, A. F., and Sundh, K. (2002) Testosterone (T) enhances apoptosis-related damage in human vascular endothelial cells. *Endocrinology* **143**, 1119–1125

26. Estrada, M., Varshney, A., and Ehrlich, B. E. (2006) Elevated testosterone induces apoptosis in neuronal cells. *J. Biol. Chem.* **281**, 25492–25501

27. Verzola, D., Gandolfo, M. T., Salvadori, F., Villaggio, B., Gianiorio, F., Traverso, P., Deferrari, G., and Garibotto, G. (2004) Testosterone promotes apoptotic damage in human renal tubular cells. *Kidney Int.* **65**, 1252–1261

28. Winiar ska, A., Mandt, N., Kamp, H., Hossini, A., Selthmann, H., Zouboulis, C. C., Blume-Peytavi, U. (2006) Effect of 5α-dihydrotestosterone and testosterone on apoptosis in human dermal papilla cells. *Skin Pharmacol. Physiol.* **19**, 311–321

29. Ulivieri, C. (2010) Cell death: Insights into the ultrastructure of mitochondria. *Tissue Cell* **42**, 339–347

30. Papadopoulou, N., Charalampoopoulos, I., Anagnostopoulou, V., Konstantinidis, G., Föller, M., Gravanis, A., Alevizopoulos, K., Lang, F., and Stournaras, C. (2008) Membrane androgen receptor activation triggers down-regulation of PI-3K/Akt/NF-κB activity and induces apoptotic responses via Bad, Fasl and caspase-3 in DU145 prostate cancer cells. *Mol. Cancer Ther.* **7**, 378–388

31. Choudhry, R., Hodgins, M. B., Van der Kwast, T. H., Brinkmann, A. O., and Boersma, W. J. (1992) Localization of androgen receptors in human skin by immunohistochemistry: implications for the hormonal regulation of hair growth, sebaceous glands and sweat glands. *J. Endocrinol. Metab.* **133**, 467–475
44. Calebiro, D., Nikolaev, V. O., Persani, L., and Lohse, M. J. (2010) Signaling by internalized G-protein-coupled receptors. Trends Pharmacol. Sci. 31, 221–228
45. Jiang, F., Zhang, Y., and Dusting, G. J. (2011) NADPH oxidase-mediated redox signaling: roles in cellular stress response, stress tolerance, and tissue repair. Pharmacol. Rev. 63, 218–242
46. Lee, S. R., Kwon, K. S., Kim, S. R., and Rhee, S. G. (1998) Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor. J. Biol. Chem. 273, 15366–15372
47. Janssen-Heininger, Y. M., Mossman, B. T., Heintz, N. H., Forman, H. J., Kalyanaraman, B., Finkel, T., Stamler, J. S., Rhee, S. G., and van der Vliet, A. (2008) Redox-based regulation of signal transduction: principles, pitfalls, and promises. Free Radic. Biol. Med. 45, 1–17
48. Saitoh, M., Nishitoh, H., Fuji, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., and Ichijo, H. (1998) Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. EMBO J. 17, 2596–2606
49. Trüeb, R. M. (2009) Oxidative stress in ageing of hair. Int. J. Trichology 1, 6–14
50. Bito, T., and Nishigori, C. (2012) Impact of reactive oxygen species on keratinocyte signaling pathways. J. Dermatol. Sci. 68, 3–8
51. Naito, A., Midorikawa, T., Yoshino, T., and Ohdera, M. (2008) Lipid peroxides induce early onset of catagen phase in murine hair cycles. Int. J. Mol. Med. 22, 725–729
52. de Rivero Vaccari, J. P., Sawaya, M. E., Brand, F., 3rd, Nusbaum, B. P., Bauman, A. J., Bramlett, H. M., Dietrich, W. D., and Keane, R. W. (2012) Caspase-1 level is higher in the scalp in androgenetic alopecia. Dermatol. Surg. 38, 1033–1039
53. Thiboutot, D., Knaggs, H., Gilliland, K., and Lin, G. (1998) Activity of 5α-reductase and 17β-hydroxysteroid dehydrogenase in the infrainfundibulum of subjects with and without acne vulgaris. Dermatology 196, 38–42
54. Abdel-Latif, A. M., Abuel-Ela, H. A., and El-Shourbagy, S. H. (2009) Increased caspase-3 and altered expression of apoptosis-associated proteins, Bcl-2 and Bax in lichen planus. Clin. Exp. Dermatol. 34, 390–395