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PPARgamma Activators Stimulate Aquaporin 3 Expression in Keratinocytes/Epidermis

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Abstract

Aquaporin 3 (AQP3), a member of the aquaglyceroporin family, which transports water and glycerol, is robustly expressed in epidermis and plays an important role in stratum corneum hydration, permeability barrier function, and wound healing. PPAR and LXR activation regulates the expression of many proteins in the epidermis and thereby can affect epidermal function. Here we report that PPARgamma activators markedly stimulate AQP3 mRNA expression in both undifferentiated and differentiated cultured human keratinocytes (CHKs). The increase of AQP3 mRNA by PPARgamma activator occurs in a dose- and time-dependent fashion. Increased AQP3 mRNA levels are accompanied by an increase in AQP3 protein in undifferentiated keratinocytes and a significant increase in glycerol uptake. Activation of LXR, RAR, and RXR also increase AQP3 mRNA levels in undifferentiated and differentiated CHKs, but to a lesser extent. PPARdelta activation stimulates AQP3 expression in undifferentiated CHKs but decreases expression in differentiated CHKs. In contrast, PPARalpha activators do not alter AQP3 expression. AQP9 and AQP10, other members of aquaglyceroporin family, are less abundantly expressed in CHKs and their expression levels are not significantly altered by treatment with LXR, PPAR, RAR, or RXR activators. Finally, when topically applied, the PPARgamma activator, ciglitazone, induces AQP3 but not AQP9 gene expression in mouse epidermis. Our data demonstrate that PPAR and LXR activators stimulate AQP3 expression, providing an additional mechanism by which PPAR and LXR activators regulate epidermal function.

Keywords

Permeability barrier function; RAR; RXR; LXR; glycerol uptake

Introduction

Aquaporins (AQPs) are a family of homologous membrane proteins, which transport both water and small neutral solutes, such as glycerol and urea, and are expressed in many mammalian tissues including skin (1, 2). To date, 13 mammalian AQPs have been identified and are categorized into two groups based on their permeability: while AQPs 1, 2, 4, 5 and 8 are primarily responsible for transporting water, AQPs 3, 7, 9 and 10 function as glycerol transporters (1, 2). AQP3 is constitutively expressed at robust levels in the stratum basale of the epidermis (3) while recent studies have demonstrated the expression of AQP-9 and -10 in mouse epidermis and human keratinocytes, respectively (4, 5). The function of AQP3 in

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Conflict of interest None

the maintenance of skin hydration has been revealed in AQP3-deficient mice, which demonstrated reduced water and glycerol permeability and decreased water holding capacity of the stratum corneum (6). Following acute barrier disruption by tape stripping, AQP3-deficient mice also show a delayed permeability barrier recovery (7). Finally, AQP3 is also important in wound healing by facilitating keratinocyte migration as a water channel and by enhancing keratinocyte proliferation and differentiation as a glycerol transporter (8). Given the critical role of AQP3 in skin biology, it is important to study the factors regulating AQP3 expression in keratinocytes/epidermis. However, how AQP3 is regulated in keratinocyte/epidermis remains largely unknown. An increase in osmolarity enhances AQP3 expression in keratinocytes (9), and topical treatment with phorbol esters increased AQP3 expression in mouse epidermis (8). Two recent reports demonstrated that AQP3 expression is regulated by calcium, 1α , 25-dihydroxyvitamin 3 (10), and retinoids (11).

Previously, we and others have demonstrated that PPAR- α , - β/δ , - γ , as well as LXR- α , and - β , are expressed in cultured human keratinocytes (CHKs) and/or murine epidermis (12-16). Activation of PPARs and LXRs regulate the expression of many important aspects of keratinocyte metabolism including a) stimulating involucrin and loricrin expression, key corneocyte envelope proteins (17-19), b) increasing the expression of transglutaminase 1, a crucial enzyme for the cross linking of cornified envelope proteins (17, 19), c) increasing the expression of Sult2B1b, which catalyzes the synthesis of cholesterol sulfate (20), d) stimulating epidermal lipid synthesis (21), and e) accelerating lamellar body secretion (21). Recently, we demonstrated that the expression of a group of lipid transporters, ABCA1, ABCG1 and ABCA12, is also markedly stimulated by LXR and PPAR activation (22-24). Since aquaglyceroporins, and in particular AQP3, play an important role in keratinocyte function, in this study we hypothesized that the expression of AQP3 is regulated by PPAR or LXR activators in keratinocytes/epidermis.

Methods

Materials

22(R)-OH-cholesterol (22R), clofibric acid (CLO), WY14643 (WY), all-*trans*-retinoic acid (ATRA), and 9-*cis*-retinoic acid (9-*cis*-RA) were purchased from Sigma (St Louis, MO). Ciglitazone (Cig) and TO 901317 (TO) were purchased from Cayman Chemical Co. (Ann Arbor, MI). Synthetic PPAR- δ activator GW 610742X (GW) and PPAR- γ activator GI 251929X (GI) were generous gifts from Dr. Tim Willson (GlaxoSmithKline). Molecular grade chemicals such as TRI Reagent were obtained from either Sigma or Fisher Scientific (Fairlawn, NJ). The iScriptTM cDNA Synthesis Kit for first-strand cDNA synthesis was purchased from BIO-RAD Laboratories (Hercules, CA). All reagents and supplies for Real-time PCR were purchased from Applied Biosystems (Foster City, CA). Primary polyclonal antibody against human AQP3 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Glycerol [$1,2,3$ - ^3H] was purchased from American Radiolabeled Chemicals, Inc., (St. Louis, MO).

Keratinocyte Culture

The second passage of human foreskin keratinocytes were seeded and maintained in 0.07 mM calcium chloride (Cascade Biologics, Portland, OR). Once the cells attached, the culture medium was switched to either low (0.03 mM) or high (1.2 mM) calcium condition, in the presence or absence of various reagents or vehicle control as described previously (22).

Quantitative PCR analysis

Quantitative real-time PCR (qPCR) was performed with a Stratagene Mx3000P as described previously (22). Briefly, following RNA isolation, cDNA was synthesized to measure the relative mRNA levels of either human AQP3, AQP9, AQP10 and cyclophilin (as internal control), or mouse AQP3, AQP9 and 36B4 (as internal control). The primer sequences for qPCR are listed in Supplemental Table 1 (Table S1). Prior to performing qPCR analysis on the samples, each primer pair undergoes several tests including amplification kinetics for the internal and target genes, melting curve analysis, and agarose gel sizing to ensure specificity. The Comparative C_T method is used for quantifying the relative expression level of target genes normalized against the internal control. The PCR reaction was performed in duplicate, with $n=3\sim4$ in each group for keratinocytes, and $n=6$ for mouse epidermis. Experiments were repeated at least once using a different batch of cells or animals to ensure reproducibility.

Western blot analysis

Western blots were performed according to the manufacturer's protocol. Briefly, the cell extract was prepared from CHKs and 50-100 μ g protein were fractionated on pre-cast Precise™ Protein Gels (Pierce, Rockford, IL) (12% or 4-20% gradient) and transferred to PVDF membrane. The proteins on the membrane were subsequently probed with polyclonal primary antibodies against human AQP3 (1: 400) or β -actin overnight at 4°C, and visualized by horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:7500) using the Enhanced Chemiluminescence (ECL) Western Blotting Detection System Kit. Membranes were then exposed to CL-XPosure film. An identical blot was probed with anti- β -actin antibody to verify the equal loading of protein.

Determination of glycerol uptake

CHKs were grown in 6-well plates and treated with 5 μ M Cig for the indicated period of time. At the end of treatment, the culture medium was aspirated and cells were incubated in plain medium containing 20 mM HEPES and 1 μ Ci/ml [1,2,3- 3 H]-glycerol (specific activity: 4 Ci/mmol) at room temperature for 10 min. Cells were then rinsed $\times 3$ in cold PBS buffer and lysed in 0.3 M NaOH. The radioactivity of the cell lysate was counted by a β -counter.

Animal treatment

Adult mouse dorsal skin was treated by topically applying either vehicle (100% acetone) or PPAR γ activator Cig (10 mM) twice a day for 3.5 days. At the end of treatment, the dorsal skin was collected and the epidermis was isolated as described previously (25). The small amount of acetone applied (~ 50 μ l) does not alter transepidermal water loss. Transepidermal water loss, SC hydration and surface pH were measured with respective probes (Tewameter, Corneometer CM825, and pH905) attached to a Courage & Khazaka MPA5 system (26).

Institutional approval

The use of human keratinocytes and animals, and all experimental procedures were approved by the appropriate committees including the Committees of Human Research at the University of California, San Francisco, and Institutional Animal Care and Use Committee of the San Francisco Veterans Affairs Medical Center.

Statistical analysis

All data are expressed as mean $\pm$ SEM. Comparison between two groups is undertaken using two-tail and unpaired t test. Comparisons within multiple groups were subjected to one-way ANOVA test, followed by Dunnett's post-hoc test to analyze the variance between two groups. Differences in values are considered significant if $p < 0.05$.

Results

AQP3 gene expression is stimulated by PPAR, LXR, RAR, and RXR activators in CHKs

We initially examined the effect of LXR, PPAR (α , δ , γ), RAR, or RXR activation on AQP3 gene expression in undifferentiated CHKs cultured in low calcium medium. As shown in Fig. 1A, AQP3 mRNA levels are markedly increased following treatment with either PPAR γ activators (Cig: 9-fold; GI: 5.7-fold), LXR activators (22R: 4.4-fold; TO: 85%, $P < 0.05$), or a PPAR δ activator (GW 610742X: 2.6-fold). In contrast, activation of PPAR α by either WY or CLO has no effect on AQP3 mRNA levels (Fig. 1A). Additionally, activation of retinoid receptors (RAR or RXR) significantly increases AQP3 mRNA levels (2.9- and 2.2-fold, respectively) (Fig. 1A), consistent with a previous report (11). Calcium (1.2 mM) alone also increases AQP3 mRNA levels (1.7-fold) (Fig. 1A).

To examine the effect of LXR, PPAR (α , δ , γ), RAR, or RXR activation on AQP3 gene expression in differentiated keratinocytes, cells were incubated with each activator in a high calcium (1.2 mM) medium. Activation of PPAR γ , LXR, RAR or RXR significantly increases AQP3 mRNA levels (4.4-, 1.5-, 3.2- and 3.3-fold, respectively) (Fig. 1B). In contrast and similar to results in undifferentiated keratinocytes, a PPAR α activator has no effect on AQP3 expression (Fig. 1B). Surprisingly, the PPAR δ activator, GW 610742X, decreases AQP3 mRNA levels by 50% in differentiated keratinocytes, an effect opposite to what was observed in undifferentiated keratinocytes.

AQP9 and AQP10 are also expressed in CHKs but are not regulated by PPARs or LXR activation

Recent reports indicated that other AQPs that transport glycerol, AQP9 and AQP10, are also expressed in keratinocytes (4, 5). Hence we next examined their expression levels and regulation by PPAR and LXR activators. Specific primers for these isoforms were designed (Supplemental Table S1), and tested for successful measurement of each isoform by RT-qPCR. AQP isoforms are amplified with C_T values of ~29-31 for AQP9 and ~34-36 for AQP10, which are much higher than for AQP3 (~22-24). (C_T values are inversely proportional to the amount of target mRNA in the sample, i.e. the higher the C_T level the lower the amount of mRNA in the sample). Furthermore, neither PPAR (α , δ , γ), LXR, RAR, nor RXR activators significantly alter AQP9 or AQP10 gene expression in undifferentiated CHKs (Supplemental Fig. S1).

These results demonstrate that although all three AQP isoforms (AQP3, 9, 10) are expressed in undifferentiated CHKs, AQP3 is the most abundant and is stimulated in response to activation of nuclear receptors including PPAR γ , PPAR δ , LXR, RAR and RXR.

PPAR γ activation increases AQP3 mRNA levels in a time- and dose-dependent manner

Since the PPAR γ activator Cig elicits a robust increase in AQP3 mRNA levels in both undifferentiated and differentiated CHKs (Fig. 1, A and B), we subsequently focused on the effect of Cig on AQP3 gene expression. The Cig-induced increase in AQP3 mRNA levels occurs in a time- and dose-dependent manner (Fig. 2). As early as 3 hrs following Cig treatment, AQP3 mRNA level increases ~68% ($p < 0.01$); at 6 hrs increases ~100% ($p < 0.01$), and this increase is sustained over an extended period of time (16-24 hrs, ~9-13 fold) (Fig. 2A). Longer incubations (≥ 72 hours) cause cell toxicity (data not shown). Cig treatment also results in a dose-dependent (2.5-10 μ M) increase in AQP3 mRNA levels (1.1-19 fold) (Fig. 2B). Higher concentration of Cig (12.5 μ M) causes cell toxicity (data not shown).

PPAR γ activation increases AQP3 protein levels

We next assessed whether PPAR γ activation also increases AQP3 expression at the protein level. As shown in Fig. 3, polyclonal antibodies against human AQP3 recognize a band migrating at ~40 KDa and a doublet at ~25-30 KDa (A), which represents both the glycosylated (40 KDa) and unglycosylated (25-30 KDa) forms of AQP3, as previously reported (27, 28). Following Cig treatment for 48 hrs, AQP3 levels, in particular the 40 KDa bands, are significantly increased (299%; $p < 0.01$) in undifferentiated keratinocytes (Fig. 3A; left panel). Since high calcium alone (Fig. 1A) or the combination of high calcium with Cig (Fig. 1B) increases AQP3 mRNA levels, we also determined AQP3 protein levels under these conditions. As shown in Fig. 3A (right panel), neither high calcium alone nor high calcium plus Cig significantly increases AQP3 protein levels. Similarly, Cig treatment for 24 hr significantly increases AQP3 protein (both 40 KDa and 30 KDa, 97% and 75%, respectively; $p < 0.05$) levels in undifferentiated but not in differentiated keratinocytes (Supplemental Fig. S2). The differential effect of the PPAR γ activator Cig on AQP3 mRNA and protein levels in undifferentiated versus differentiated keratinocytes is unclear.

PPAR γ activation increases glycerol uptake

AQP3 is well-known for transporting glycerol (1). We therefore investigated whether PPAR γ activation enhances glycerol uptake in CHKs. Keratinocytes cultured in low levels of extracellular calcium (0.03mM) remain in a proliferative state, and over time there is a significant increase in [3 H]-glycerol uptake (24hr, 32%; 48hr, 1.1-fold; 72hr, 1.5-fold, respectively). Cig treatment increases [3 H]-glycerol uptake at all time points (24hr, 79%; 48hr, 1.7-fold; 72hr, 2.9-fold, respectively) (Supplemental Fig. S3A). In parallel, there is a time-dependent increase in AQP3 mRNA levels in both vehicle control and Cig treated keratinocytes (Supplemental Fig. S3B). As expected, Cig increases AQP3 mRNA levels at all time points (note that in these studies a low dose of Cig, 2.5 μ M, was used that did not induce toxicity with extended incubation).

Topical treatment with a PPAR γ activator increases AQP3 mRNA levels in mouse epidermis

We next determined whether PPAR γ activation increases AQP3 expression *in vivo*. Initially we examined the basal mRNA levels of the AQP isoforms in mouse epidermis. Consistent with the expression pattern in CHKs, AQP3 is expressed at high levels (C_T : 20~22) in epidermis while AQP9 is modestly expressed (C_T : 27~28) (Supplemental Table 2). (Of note the mouse AQP10 gene has not been cloned, so no data is available for AQP10 mRNA levels in mouse epidermis). Furthermore, when topically applied on skin for 3.5 days, the PPAR γ activator Cig significantly increases AQP3 mRNA levels (~2.2-fold) in mouse epidermis (Fig. 4). In contrast, mAQP9 mRNA levels remain unchanged following Cig topical treatment (data not shown), consistent with the data in CHKs. Together, our results demonstrate that PPAR γ activation differentially up-regulates AQP3 gene expression both *in vitro* in cultured human keratinocytes and *in vivo* in mouse epidermis. Stratum corneum pH, basal trans-epidermal water loss and stratum corneum water-holding capacity were all similar in PPAR γ versus vehicle (control) mice (data not shown).

Discussion

The present study demonstrates that PPAR γ activators stimulate the expression of AQP3 in both undifferentiated and differentiated human keratinocytes. Additionally, topical treatment of mouse skin with PPAR γ activators increases AQP3 mRNA levels in the epidermis. Activators of PPAR δ , LXR, RAR, and RXR also stimulate AQP3 expression but the increase is less than that observed with PPAR γ activators. In contrast, PPAR α activators had no effect on AQP3 expression. Notably treatment with PPAR, LXR, RAR, and RXR

activators did not significantly alter the expression of AQP9 and 10, two additional glycerol transporters that are also present in keratinocytes. The relative abundance of AQP9 and 10 is much less than AQP3 in both human keratinocytes and mouse epidermis (Supplemental Table S2). The role of AQP9 and 10 in the epidermis is unknown.

Much of what is known regarding the function of AQP3 in the epidermis has been elucidated by studies of mice deficient in AQP3 (AQP3 knock-out mice) by Verkman's laboratory. These mice have dry and rough skin and a decrease in stratum corneum hydration (2, 6). The reduced stratum corneum hydration correlates with a decrease in glycerol in the stratum corneum and can be corrected by systemic glycerol administration (2, 29). In addition, these animals have delayed wound healing. The delay in wound healing is accounted for by both decreased keratinocyte migration and decreased keratinocyte proliferation (2, 8). Finally, AQP3 mice have a delay in the recovery of permeability barrier function following acute barrier disruption (2, 7). This abnormality can be corrected by providing additional glycerol, which is thought to facilitate lipid synthesis and/or keratinocyte proliferation (2). Thus, AQP3 has multiple key functions in the epidermis.

As discussed in detail in the introduction, PPARs and LXR are expressed in the epidermis (13, 14, 16), and PPAR and LXR activators have multiple effects on keratinocytes (16). Activation of PPARs and LXR results in the increased expression of a large number of key proteins in keratinocytes including involucrin, loricrin, transglutaminase 1, ABCA12, etc. (12, 16, 17, 19, 23). These changes result in enhanced keratinocyte differentiation (16, 30), accelerated permeability barrier repair after acute disruption (31), improved wound healing (32-34), decreased adverse effects of glucocorticoids (35), and a decreased inflammatory response (16, 36, 37). The results of this study demonstrate that an increase in the expression of AQP3 can be added to the growing list of important proteins that are regulated in keratinocytes/epidermis by PPAR and LXR activation. It is likely that the increase in AQP3 expression contributes to the effects of PPAR and LXR on epidermal function. However, it should be recognized that activation of PPARs and LXR have multiple actions (16), and thus increasing AQP3 is likely to be only a part of an orchestrated effect. For example, studies by our laboratory have shown that PPAR and LXR activation accelerate permeability barrier repair by stimulating epidermal lipid synthesis (21), increasing ABCA12 and ABCG1 expression (23, 24), which would facilitate the uptake of lipids into lamellar bodies, increasing lamellar body secretion (21), and facilitating extracellular lipid processing in the stratum corneum (21). In addition, PPAR and LXR activation stimulates keratinocyte differentiation, which would facilitate the formation of corneocytes needed to provide a scaffold for the lipid membranes that mediate permeability barrier function (16). Finally, PPAR and LXR activators stimulate SULT2B1b expression (20), the key enzyme in the synthesis of cholesterol sulfate, which would increase stratum corneum cohesion and thereby facilitate barrier function. Thus, the increase in AQP3 expression can be added to the large number of changes that are induced by activation of PPARs and LXR that could have beneficial effects on epidermal function.

The mechanism by which PPAR γ activators stimulate AQP3 expression remains unclear. Nonetheless, there are at least two lines of evidence indicating that this is likely to be an indirect event: First, time-course experiments (Fig. 2A) demonstrate that the increase in AQP3 mRNA levels is a delayed effect. With nuclear hormone receptor activation, direct effects typically occur more rapidly. Second, from both published (38) and our own promoter analysis of human AQP3 gene (unpublished observation), there is no obvious PPAR binding site (PPRE) in the proximal promoter region of AQP3. Future studies are necessary to decipher how PPAR γ activators induce AQP3 expression in human keratinocytes.

In conclusion, the present study demonstrates that PPAR γ , PPAR δ , LXR, RAR, and RXR activators can increase the expression of AQP3 in keratinocytes. Given the key functions of AQP3 in epidermal biology, it is possible that the use of these activators to increase AQP3 could be helpful in the prevention or treatment of skin disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AQP3	aquaporin 3
ATRA	all- <i>trans</i> retinoic acid
CHKs	cultured human keratinocytes
Cig	ciglitazone
CLO	clofibrate
9-<i>cis</i>-RA	9- <i>cis</i> -retinoic acid
GI	GI 251929X
GW	GW 610742X
LB	lamellar body
LXR	liver X receptor
PPAR	peroxisome proliferators-activated receptor
22(R)	22(R)-OH-cholesterol
RAR	retinoic acid receptor

RXR	retinoic X receptor
TO	TO 901317
SC	stratum corneum
WY	WY14643

Fig. 1. PPAR, LXR, RAR, or RXR activators stimulate AQP3 gene expression

(A) CHK were incubated with either vehicle or activators of PPAR α (WY 20 μ M; CLO 400 μ M), PPAR δ (GW 610742X 8 μ M), PPAR γ (Cig 5 μ M, GI 10 μ M), LXR (22R 10 μ M; TO 10 μ M), RAR (ATRA 1 μ M), or RXR (9cis-RA 1 μ M) in 0.03 mM calcium medium for 24 hrs. One set of cells were cultured in high calcium (1.2 mM) medium. (B) Cells were incubated in high calcium medium with either vehicle or activators of PPAR α (WY), PPAR δ (GW 610742X), PPAR γ (Cig), LXR (22R), RAR (ATRA), or RXR (9cisRA) for 24 hrs. RT-qPCR was performed to measure mRNA levels of AQP3 and cyclophilin (as internal control). Data are expressed as percentage of vehicle control and presented as mean $\pm$ SEM (n = 4). Similar results were obtained when the experiment was repeated twice with different batches of keratinocytes. * P < 0.05, ** P < 0.01, *** P < 0.001.

Fig. 2. Activation of PPAR γ increases AQP3 mRNA levels in a time- and dose-dependent fashion CHKs were incubated with either Cig (5 μ M) or vehicle in 0.03 mM calcium for various periods of time (0, 3, 6, 16, and 24 hrs) (**A**). Alternatively, cells were incubated with various concentrations of Cig (0, 2.5, 5, 7.5, 10 μ M) or vehicle for 24 hrs (**B**). AQP3 and cyclophilin mRNA levels were determined by RT-qPCR. Data are expressed as percentage of control and presented as mean $\pm$ SEM (n = 3). For the time course studies, data are presented as percentage of vehicle control (in the absence of Cig) for each matched time point. * P < 0.05, ** P < 0.01, *** P < 0.001.

Fig. 3. PPAR γ activator increases AQP3 protein levels in undifferentiated but not in differentiated keratinocytes

CHKs were incubated with either Cig (5 μ M) or vehicle in either 0.03 mM or 1.2 mM calcium medium for 48 hrs. The cell lysate was prepared and subjected to Western blot analysis to determine AQP3 or β -actin protein level as described in *Methods*. A representative blot is shown (**A**). The densitometry ratio of AQP3 over β -actin is expressed as percentage of control, plotted and presented as mean $\pm$ SD ($n = 4$ in each group) (**B**). Similar results were obtained when the experiment was repeated twice with different batches of cells, using 4-20% gradient gel. ** $P < 0.01$.

Fig. 4. Topical treatment with a PPAR γ activator increases AQP3 mRNA levels in mouse epidermis

Hairless mice were topically treated with either the PPAR γ activator Cig (10 mM) or vehicle for 3.5 days, and the epidermis was isolated for measuring AQP3 or 36B4 (as internal control) mRNA levels by RT-qPCR as described in *Methods*. Data are expressed as percentage of vehicle control and presented as mean $\pm$ SEM (n = 6). Representative graph is shown from 2 different sets of animals with similar results. * $P < 0.05$.