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Activity of aquaporin 3 (AQP3) and Notch1 genes via the aryl hydrocarbon receptor (AhR) in human immortalized keratinocytes exposed to 6-formylindolo [3,2-b]carbazole (FICZ) and benzo[a]pyrene (BaP)

TESIS QUE PRESENTA

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PARA OBTENER EL GRADO DE DOCTOR

EN CIENCIAS BIOMÉDICAS BÁSICAS

DIRECTOR DE TESIS

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Benzo(a)pyrene Induces Changes in AQP3-Notch1 Axis Via Activation of Aryl Hydrocarbon Receptor (AhR) in Hacat Cells

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Abstract

Background: Polycyclic aromatic hydrocarbons (PAHs) have been related to skin disorders such as aging, pigmentary illness, acne, and cancer. However, the precise mechanisms by which PAHs exposure harms the skin are still unclear. Objective: Therefore, the aim of this study was to evaluate the effect of Benzo(a)pyrene (BaP) on expression levels of AQP3 and Notch1 genes in Hacat cells exposed “in vitro” to the chemical compound. Methods: Hacat cells were exposed to increasing concentrations of BaP (1.0, 1.5, 2.0, 2.5, 3.0, and 4.0 µM) at different exposure times (1-4 days). After treatments, cell viability was measured using the Resazurin assay. Also, a real-time PCR technique was developed to assess the gene expression levels of CYP1A1, AQP3 and Notch1. Finally, to evaluate the participation of the AhR signaling pathway on gene expression levels of AQP3 and Notch1, a small interference RNA (siRNA) specifically targeting AhR was employed. Results: Non-cytotoxic effects were observed after Hacat cells were treated with increasing concentrations (1.0, 1.5, 2.0, 2.5, 3.0, and 4.0 µM) of BaP at different exposure times (1-4 days) according to the Resazurin assay. Also, a significant ($p<0.05$) over-expression of CYP1A1 mRNA in Hacat cells exposed to BaP compared with expression levels detected in the unexposed (control and DMSO) Hacat cells was detected. CYP1A1 mRNA was used as an activation marker of AhR. Similarly, an augmented expression level of AQP3 and Notch1 was sensed in exposed (BaP) Hacat cells contrasted to levels of expression in untreated controls ($p<0.01$; $p<0.001$), that effect was dependent on the activation of AhR as shown by RNA interference (specific AhR siRNA) ($p<0.01$), indicating that both genes are

downstream of AhR signaling pathway. Finally, regulation of Notch1 mRNA expression levels dependent of AQP3 was sensed in this study. Conclusion: The results presented suggest that AQP3 and Notch1, two important genes that participate in proliferation/differentiation progressions of skin tissue, are regulated by AhR activation in Hacat cells exposed to BaP. To our knowledge, this is the first study that showed the interplay between AhR, AQP3, and Notch1 signaling pathways in Hacat cells exposed to BaP.

Keywords: AQP3. Benzo[a] pyrene, Hacat cells, Notch1, PAHs

Introduction

Polycyclic aromatic hydrocarbons (PAHs) belong to a group of persistent organic pollutants originated from deficient combustion of a great variety of organic materials, including fossil fuels, wood, coal, waste, tobacco, among others (IARC), 2019). Exposure to PAHs has been related to a wide variety of harmful consequences on human health such as skin, immune, developmental, and cardiovascular toxicity (Kim et al., 2013; Lin et al., 2018; O´Driscoll et al., 2018; Ochoa-Martinez et al., 2017; Soeur et al., 2017). For example, recent investigations have described strong associations between PHAs exposure and several types of cancer (breast, lung, skin, etc) in human populations (Fent et al., 2014; Moorthy et al., 2015; Stults and Wei, 2018). Among all PAHs (even hundreds of compounds), sixteen PAHs have been identified by the US EPA (United States Environmental Protection Agency USEPA) as a priority, as they display clear evidence of mutagenicity and genotoxicity in humans and other organisms as well as their persistence and prevalence in the environment (Hussar et al., 2012). In this line, benzo[a]pyrene (BaP) is recognized as a representative compound of that group (PAHs), as well-documented carcinogenicity in humans has been established (Humans, 2012). Therefore, BaP has been accepted as an excellent chemical model for the study of PAHs. BaP is an effective activator of the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor, extensively expressed in a wide diversity of cells including skin keratinocytes (Carlstedt-Duke, 1979; Harper et al., 2006). The AhR activation regulates transcription of responsive genes such as xenobiotic-metabolizing enzymes (i.e. cytochrome P450 1A1 (CYP1A1) and

CYP1A2) (Costa et al., 2010; Skupinska et al., 2009). Besides, accumulating scientific information suggests that the carcinogenic effects generated by BaP are mediated, at least in part, by activation of aryl hydrocarbon receptor (Bjelogrlic et al., 1994; Colapietro et al., 1993; Shimizu et al., 2000).

Emerging investigations suggest that environmental pollutants have a variety of detrimental effects on skin health (Wu et al., 2016). In this regard, it has been showing that chronic dermal exposure to PAHs may be related to skin disorders such as aging, pigmentary illness, acne, and cancer (Krutmann et al., 2014). However, the precise mechanisms by which PAHs exposure harms the skin are still unclear. Recently, Hieda et al. (2020) have found an association between coarse air particulate matter (PM) exposure and modifications in markers (AQP3 and Notch1 proteins) of proliferation/differentiation in a reconstructed human epidermis model, an exciting finding, as PAHs are the most important components of PM. Besides, Hieda et al. suggest that alterations observed in assessed proteins could eventually compromise the skin integrity and promoting or intensifying several skin illnesses (Hieda et al., 2020), as AQP3 and Notch1 proteins in the skin play an important role in maintaining homeostasis in the tissue (Bollag et al., 2007; Guo et al., 2013; Rangarajan et al., 2001; Zheng and Bollag, 2003). Impairment of either AQP3 or Notch1 signaling pathways has been related to skin diseases such as psoriasis, atopic eczema, atopic dermatitis, and cancer (Abdou et al., 2012; Dumortier et al., 2010; Hara-Chikuma and Verkman, 2008; Lee et al., 2012a; Nakahigashi et al., 2011; Nicolas et al., 2003).

Therefore, the aim of this study was to evaluate the effect of BaP on expression levels of AQP3 and Notch1 genes in Hacat cells exposed “in vitro” to the chemical compound.

Materials and methods

Cell culture

Hacat cells (human epidermal keratinocyte line) were maintained in Epilife culture medium (EDGS growth supplement; Life technologies, Carlsbad, CA) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin at 37 ºC in a humidified atmosphere of 5 % CO2 and 95 % air.

Cell viability assay

Resazurin assay was used to determine Hacat cells viability as stated by Riss et al (2016) (Riss et al., 2016). Hacat cells were seeded in 96 well plates (0.5 x 104 cells/mL) and incubated for 24 hours at 37 °C and 5 % CO2 atmosphere. After the incubation period, cells were washed with phosphate-buffered saline solution (PBS), and a fresh culture medium was added. Then, Hacat cells were dosed with increasing concentrations (1.0, 1.5, 2.0, 2.5, 3.0, and 4.0 µM) of benzo[a]pyrene (Sigma-Aldrich, St Louis, MO, USA) for four days. After treatments, cells were washed with PBS and freshly culture medium plus resazurin reagent (30 µg/mL; Sigma-Aldrich, St Louis, MO, USA) was added to each well and incubated for two hours in a humidified atmosphere of 5 % CO2 and 95 % air (Riss 2013). Fluorescent

measurements with an excitation wavelength λ=560 nm and an emission wavelength λ=590 nm were performed to determine the degree of metabolism of resazurin in a Spectrofluorometer Synergy H1 (BioTek Instruments Inc. Winooski, VT, USA). Untreated Hacat cells were used as negative control, cells dosed with 0.5 % of DMSO were used as vehicle control, and cells treated with H2O2 (0.6 %) were set as the positive control.

Total mRNA isolation

Hacat cells were seeded in 24 well microplates and at 90% confluent monolayers were exposed to 2.5 µM of BaP for three days at 37 °C in a humidified atmosphere of 5 % CO2 and 95 % air. After treatment, Hacat cells monolayers were washed twice with PBS and total RNA was extracted using TRIZOL® reagent (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA) following the manufacturer´s instructions. Spectrophotometric analysis at 260 nm was performed on an Eon Microplate Spectrophotometer (BioTek) to assess final RNA concentration. The obtained samples were stored at -80°C until their analysis.

Gene expression

Obtained RNA was reverse transcribed using a Reverse Transcription Kit and OligodT primers (Applied BioSystems, Foster City, CA, USA) according to the manufacturer´s protocol. Subsequently, the product (cDNA) was adjusted to a concentration of 100 ng/µL and used for the gene expression determination.

GAPDH, AHR, CYP1A1, AQP3, and NOTCH1 were amplified by a real-time PCR detection system using specific TaqMan® probes (Applied BioSystems, Foster City, CA, USA). The thermal cycling process was established as follows: a preliminary denaturation step at 95 °C for 10 min, 40 cycles of PCR amplification at 95 °C (15 s), and 60 °C (60 s). The relative expression level of each candidate gene was individually normalized to the housekeeping gene (GAPDH) and expressed as $2^{-\Delta\Delta Ct}$.

Transfection with specific small interference RNA

Hacat cells were transfected with siRNA specifically targeting AhR (ThermoFisher Scientific, Waltham, MA, USA), AQP3 (Santa Cruz Biotechnology, Dallas, Texas, USA), or control (ThermoFisher Scientific, Waltham, MA, USA) using the siLentFectTM Lipid Reagent (BioRad, Hercules, CA, USA) according to the manufacturer's instructions. Briefly, Hacat cells cultured in 24 well plates and at 50-60 % confluence were incubated with a mixture of 0.25 µL SiLentFect transfection lipid reagent containing 10 nM of respective siRNA in Opti-MEM reduced serum medium GlutaMax supplemented, purchased from Gibco (ThermoFisher Scientific Waltham, MA, USA) to a final volume of 25 µL per condition. After an incubation period (4 hours), siRNA-transfected Hacat cell monolayers were washed with PBS and replaced with fresh EpiLife supplemented medium. Once the cells reached 90% of confluence, the cells were exposed to BaP 2.5µM for three days. Then, the cells were lysed for RNA extraction and analyzed for AhR, AQP3, Notch1, and GAPDH gene expression.

Statistics

Prism software was used for statistical analyses (GraphPad Software 5.0, La Jolla, CA). The data were analyzed with the Shapiro-Wilk test to evaluate the normal distribution of the data. Data are present as mean ± standard error of the mean from at least three different experiments. When data were not normal, non-parametric (Friedman test) test was performed followed by Dunn´s post-hoc test to compare groups. Normal data were evaluated using t-student test. Values of $p \leq 0.05$ were considered significant.

Results

Cell viability

Hacat cells were exposed to increasing concentrations of BaP (1.0, 1.5, 2.0, 2.5, 3.0, and 4.0 µM) for 4 days and cell viability was assessed using the Resazurin assay as stated in materials and methods. Figure 1 shown Hacat cells viability after BaP treatments. According to ISO 10993-5, in “in vitro” cytotoxicity tests for biological devices or materials (International Organization for Standardization, 2009), cell viability above 80% are considered as non-cytotoxic. In this regard, non-cytotoxic effects of BaP were observed after Hacat cells were dosed with 1.0, 1.5, 2.0, 2.5, 3.0, and 4.0 µM (viability cell higher than 80 %) for four days. Because all the tested concentrations of BaP were harmless, to next experiments 2.5 µM of BaP and three days of exposure were used. We decided to establish this condition because at this

concentration, BaP has demonstrated to induce gene expression of AQP3 and Notch1 in different cellular models (Bui, et al 2015).

Gene expression (CYP1A1, AQP3, and Notch1)

The effect of BaP 2.5 μM on CYP1A1 mRNA expression in Hacat cells “in vitro” as an activation marker of AhR was assessed. It was found a significant over-expression of CYP1A1 mRNA in Hacat cells exposed to BaP 2.5 μM compared to the expression levels detected in the unexposed (control and DMSO) Hacat cells (Figure 2a). Also, a significant increment in the expression levels of AQP3 mRNA (p□0.001; Figure 2b) and Notch1 mRNA (p□0.05; Figure 2c) was detected in Hacat cells exposed “in vitro” to BaP 2.5 μM for 3 days contrasted to expression levels found in the control cells (control and DMSO).

AhR siRNA transfection

Next, to investigate the contribution of AhR activation on AQP3 and Notch1 mRNA expression levels in Hacat cells exposed “in vitro” to BaP 2.5 μM for 3 days, a specific AhR silencing RNA was used (AhR siRNA). As shown in Figure 3a, an effective block of the AhR expression ($p<0.001$) was achieved after Hacat cells were treated with the AhR siRNA. Besides, expression levels of AQP3 mRNA (Figure 3b) and Notch1 mRNA (Figure 3c) were significantly downregulated in Hacat cells dosed with BaP 2.5 μM for 3 days and the AhR siRNA, which indicates that both genes (Notch1 and

AQP3) are regulated by the AhR activation (AQP3 $p< 0.01$; Notch1 $p< 0.001$) in the studied cell model.

AQP3 – Notch1 axis communication

To elucidate if AQP3 has a negative regulation on Notch1 mRNA expression as informed early by Guo et al. (2013), we decided to knock-down the AQP3 gene with a specific siRNA (AQP3 siRNA). Figure 4a shows the successful knock-down of AQP3 mRNA expression when Hacat cells were treated with the AQP3 siRNA. Also, significant overexpression of Notch1 mRNA expression was distinguished (Figure 4b) after AQP3 was silenced.

Finally, expression levels of Notch1 mRNA were assessed when Hacat cells were co-exposed to AQP3 siRNA and BaP 2.5 µM for 3 days. As shown in Figure 4c, an exacerbated overexpression of Notch1 mRNA expression level was registered in presence of BaP and AQP3 siRNA when compared to control cells ($p<0.001$).

Discussion

Though BaP is the model compound used for studying the carcinogenic properties of polycyclic aromatic hydrocarbons (PAHs), the effects of BaP on the skin tissue have been poorly characterized, and molecular responses are usually extrapolated from animal studies (Bjelogrlic et al., 1994; Briedé et al., 2004; Colapietro et al., 1993; Evans et al., 2004). In this study, AQP3 and Notch1 mRNA expression levels were evaluated in Hacat cells (an experimental cell model extensively used for

studies related to skin biology, proliferation, and differentiation) exposed to BaP 2.5 µM for three days. AQP3 is an aquaglyceroporin involved in the transport of water, urea, and glycerol across biological membranes in mammalian skin (Blaydon and Kelsell, 2014; Ma et al., 2002). Besides, recent evidence suggests a pivotal role of AQP3 in skin cancer disorders (Hara-Chikuma and Verkman, 2008). The Notch proteins are type-1 trans-membrane receptors and upon interaction with membrane-bound ligands, Notch is released from the plasma membrane and translocated to the cellular nucleus acting as a transcription factor (Watt et al., 2008). In mammalian skin, Notch-mediated signaling exerts a critical pro-differentiation and tumor-suppressing function (Moriyama et al., 2008; Nicolas et al., 2003). Recently, an increasing amount of scientific evidence has demonstrated a reciprocal negative feedback loop between AQP3 and Notch signaling (Guo et al., 2013).

In this study, AQP3 mRNA was upregulated in Hacat cells exposed to BaP 2.5 µM for three days (Figure 2b), that effect resulted from the activation of AhR as shown by RNA interference (Figure 3b). Similar results were detected in different cellular models exposed to AhR agonists (Bui et al., 2015; Wu et al., 2014). Bui et al (2015), have demonstrated overexpression of AQP3 mRNA and protein in human HepG2 cells treated with the environmental pollutant, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Also, an increment in the expression levels of AQP3 mRNA in several tissues extracted from murine models exposed to TCDD was found (Bui et al., 2015). The effects found in the Bui et al. (2015) study were associated with the AhR activation. Hieda et al., (2020) have found a significant increase in the AQP3 expression level in reconstructed human epidermis exposed to different

concentrations of coarse air particulate matter (PM) “in vitro” (Hieda et al., 2020). The PM is an exceptional sink of PAHs including BaP (Schwarse et al., 2006). Accumulating evidence indicates that AQP3 plays a key role in several processes involved in keratinocyte function, and irregularities in the expression and function of this channel have been detected in some human skin diseases such as psoriasis, atopic dermatitis, atopic eczema, vitiligo, and cancer (Hara-Chikuma and Verkman, 2008; Hara-Chikuma et al., 2015; Kim and Lee, 2010; Lee et al., 2012b; Nakahigashi et al., 2011; Olsson et al., 2006; Voss et al., 2011).

Also, after Hacat cells were exposed to BaP 2.5 µM for three days, overexpression of Notch1 mRNA was detected (Figure 2c), the change in the expression level of Notch1 was related to the AhR activation as demonstrated by RNA interference (Figure 3c). In agreement with our results, Boverhof et al. (2006) found an increased expression level of Notch1 mRNA in hepatic tissue of Sprague Dawley rats and C57BL/6 mice dosed with different concentrations of TCDD (Boverhof et al., 2006). Similar results were found by Kaminska, et al (2018) in Sertoli cells cultures exposed to 5x10-5M and 1x10-4M of the plastic derived compound bisphenol A and dibutyl phthalate respectively, where an upregulation of Notch1 expression was registered and related to the potential effect of both chemicals to promote juxtacrine communication in male gonads (Kaminska et al., 2018). Both compounds have shown induce AhR activation. Similarly, the treatment of reconstructed human epidermis with PM “in vitro” overexpressed the Notch 1 mRNA (Hieda et al., 2020). The real implication of Notch1 in carcinogenic processes in human skin has been not completely elucidated. However, recent experimental studies have suggested

that Notch signaling functions as a tumor-suppressor gene in mammalian skin (Cialfi et al., 2014; Nicolas et al., 2003). Also, recent results indicate that an unusual expression of Notch1 is not involved in the initiating event of multistage skin carcinogenesis but acts as a skin cancer-promoting event (Cialfi et al., 2014). Our results demonstrated overexpression of Notch1 mRNA in Hacat cells exposed to BaP. But, the real significance on skin homeostasis needs to be elucidated.

Finally, a regulation of Notch1 mRNA expression levels dependent of AQP3 was sensed in this study, as an increment in the Notch1 mRNA expression was observed when AQP3 was silenced (Figure 4b). Besides, the effect of AQP3 on Notch1 mRNA expression levels was intensified when Hacat cells were co-treated with BaP 2.5 µM (Figure 4c). In this line, early studies have suggested a reciprocal negative feedback loop between AQP3 and Notch 1. For example, Guo et al (2013), found that when the expression level of AQP3 was elevated, Notch 1 expression levels were diminished in a keratinocyte cell model (Guo et al., 2013). Besides, a similar regulation between both molecules (AQP3 and Notch 1) has been observed in several skin diseases such as psoriasis and atopic dermatitis (Abdou et al., 2012; Melnik, 2015). In our study, when AQP3 was silenced, an increment in Notch1 mRNA expression was observed, which could be explained by the negative regulation that AQP3 exerts over Notch1 gene expression. However, the real significance of these findings demands to be elucidated.

Interestingly, an increasing amount of scientific investigations have demonstrated interplays and crosstalk between AhR and several intracellular signaling pathways (as observed in this research), including NF-κB, Nrf2, Rb/E2F, and Sp1, as well as

other transcription factors, such as the androgen receptor, and the estrogen receptors (ERα and ERβ) (Boverhof et al., 2008; Joo et al., 2013; Luecke et al., 2010; Puga et al., 2009; Ye et al., 2019). For example, an interplay between the AhR and the ERα signaling pathways has been detected for several years (L´Héritier et al., 2014). ERα belongs to the nuclear receptor family of transcription factors and is involved in the regulation of cellular proliferation in response to 17β-estradiol, for example during mammary gland development (Helle et al., 2016; Safe et al., 2000). Although an interaction between AhR, AQP3 and Notch1 signaling pathways was observed in this investigation in Hacat cells treated with BaP, more studies are necessary to elucidate the real significance of the findings on skin health.

Conclusion

The contribution of the AhR signaling pathway to skin homeostasis has been well established. However, the diverse molecular mechanisms involved in that processes remain to be fully elucidated. The results presented herein suggest that AQP3 and Notch1, two important genes that participate in proliferation/differentiation progressions of skin tissue, are upregulated by AhR activation in Hacat cells exposed to BaP. To our knowledge, this is the first study that showed the interplay between AhR, AQP3 and Notch1 signaling pathways in Hacat cells exposed to BaP. Nonetheless, the impact on skin homeostasis was not clarified, therefore, further investigations are required to understand the influence of results found in this investigation on skin tissue health.

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Declaration of interest

The authors declare no conflict of interest

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Figures legends

Figure 1. Cell viability assay

Hacat Cells were dosed with increasing concentrations of BaP (1.0, 1.5, 2.0, 2.5, 3.0, and 4.0 µM). Then, Resazurin assay was performed at 1, 2, 3, and 4 days of exposure. The percentage of viable cells was calculated and expressed as the average of three independent experiments, each condition in quadruplicate.

Figure 2. Gene expression

Hacat cells were exposed to BaP 2.5 µM for three days. a) CYP1A1 gene expression. b) AQP3 gene expression. c) Notch1 gene expression; GAPDH was used as a house-keeping gene; *P<0.05, **P<0.01 and ***P<0.001 versus untreated controls. The results are representative of five independent experiments.

Figure 3. Effect of AHR-siRNA on AhR and NOTCH1 expression

Hacat cells were transfected with Control-siRNA and AhR-siRNA with the lipid-cationic reactant siLenfect. Lysates were obtained to measure gene expression. a) AhR, b) AQP3, and c) Notch1 gene expression levels in presence of AhR-siRNA and control-siRNA. Gene levels were normalized to GAPDH expression levels; **P<0.01, and ***P<0.001 versus untreated controls. The results are representative of three independent experiments.

Figure 4. AQP3 and Notch1 axis

Hacat cells were transfected with Control-siRNA and AQP3-siRNA with the lipid-cationic reactant siLenfect. Lysates were obtained to measure gene expression. a) AQP3, b) NOTCH1 gene, c) NOTCH1 gene expression in presence of AQP3-siRNA exposed to BaP 2.5µM; AQP3 and NOTCH1 gene levels were normalized to GAPDH expression levels; *P<0.05, **P<0.001, and ***P<0.001 versus untreated controls. The results are representative of five independent experiments.

Figures

Fig. 1

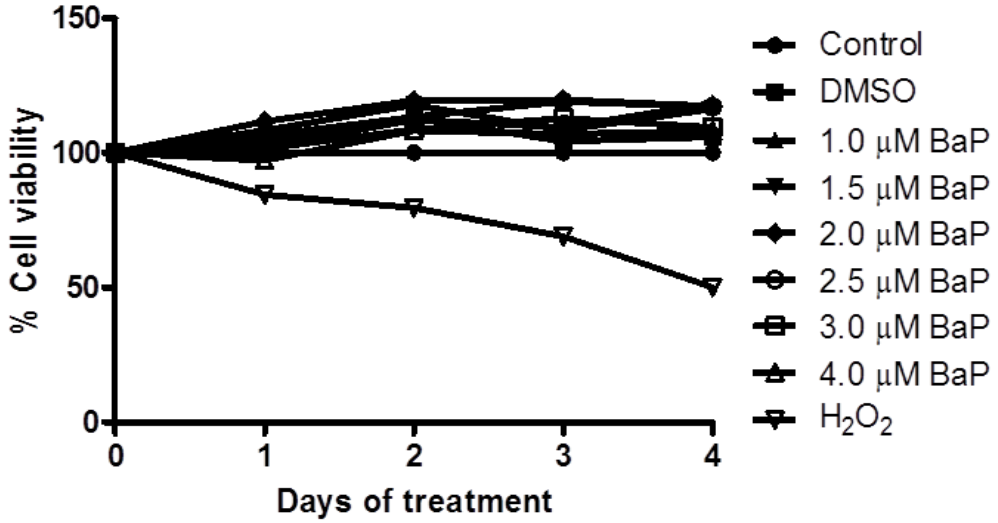
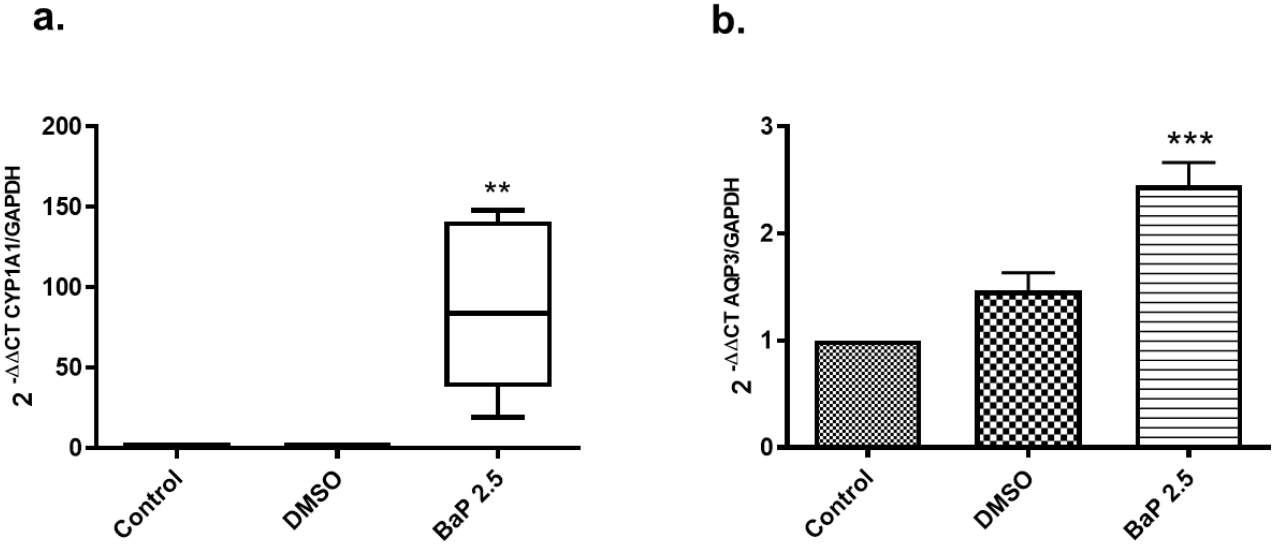


Fig. 2



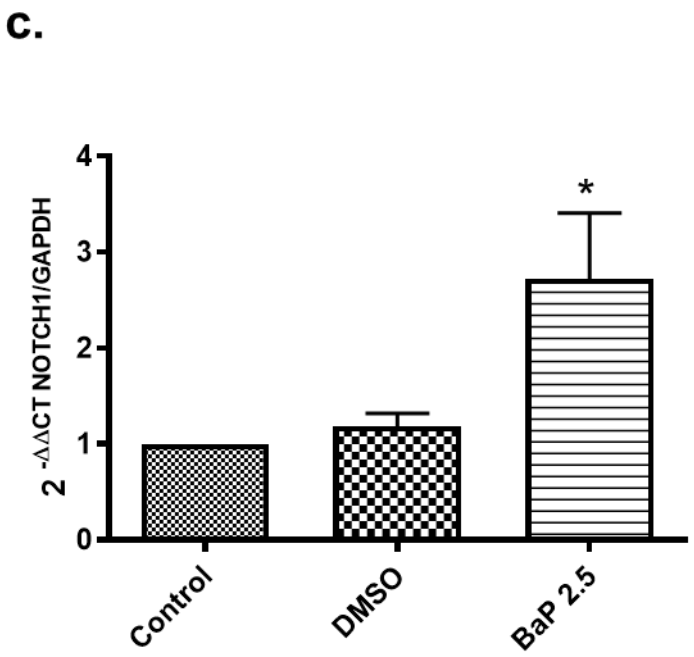

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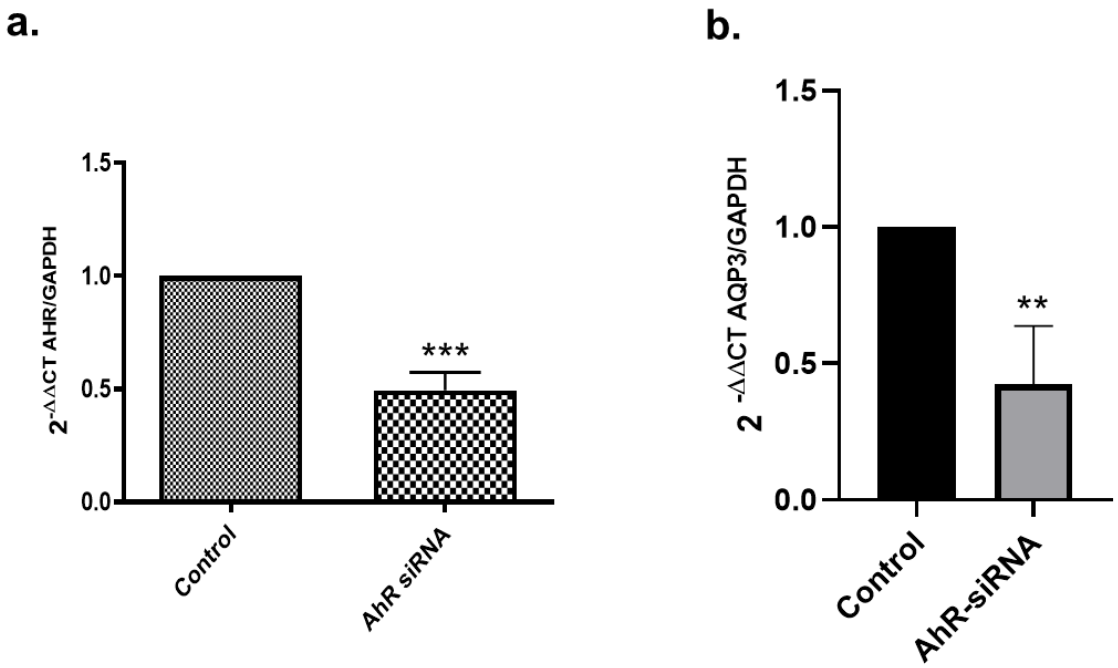
Fig. 3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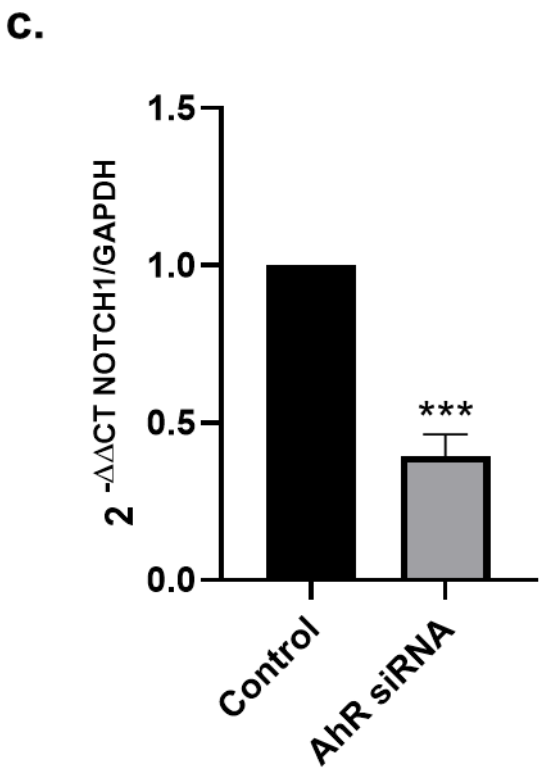
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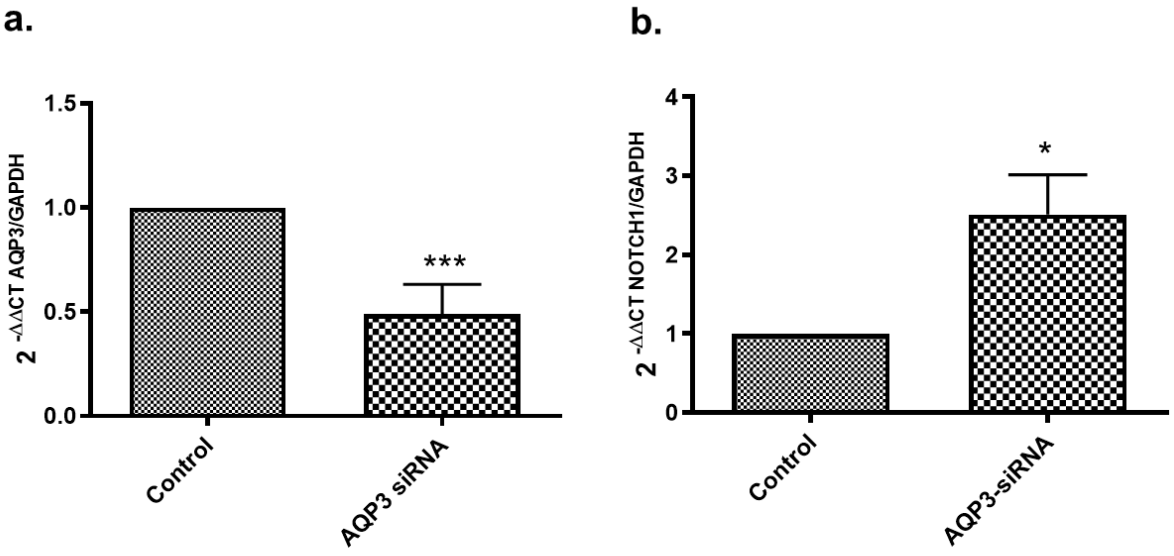
Fig. 4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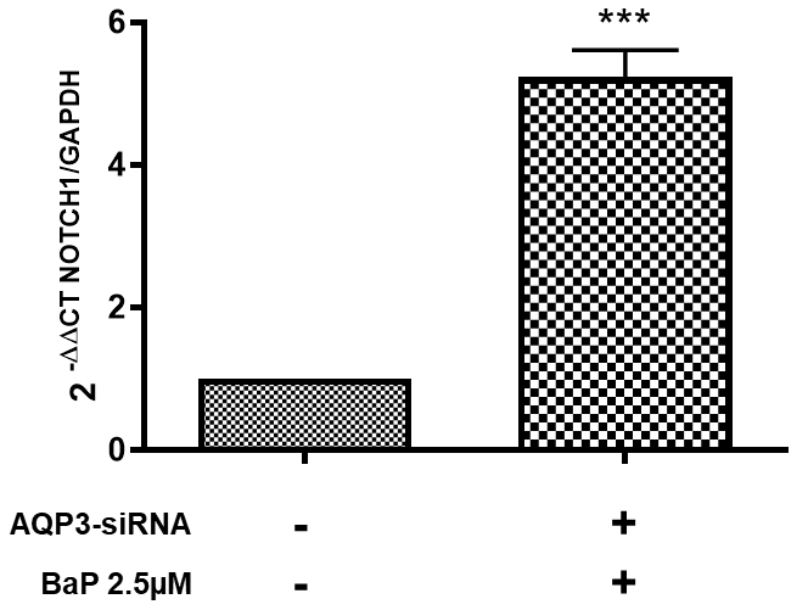

Skin disease related protein aquaporin-3 (AQP3) is downregulated by 6-formylindolo[3,2-b]carbazole exposure in a model of human immortalized keratinocyte cell line (Hacat)

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Abstract

Background: Several skin diseases are related to oxidative stress induced by environmental factors, especially pollutants. In the tissue, activation of signaling proteins like aryl hydrocarbon receptor (AhR) promote the modulation of many genes like aquaporin 3, which is involved in proliferation, hydration, migration and has found altered in many clinical skin traits. In the present study we evaluated the modulation of the AQP3 in presence of 6-formylindolo[3,2-b]carbazole (FICZ) under benzo(a)pyrene (BaP) mRNA AQP3 gene overexpression, an important member of the polycyclic aromatic hydrocarbon family of pollutants. Objective: The aim of this study was to evaluate the influence of FICZ on AQP3 gene expression (a gene involved in cellular skin homeostasis). Methods: Hacat cells were exposed to FICZ (1, 10, and 100 nM) and benzo[a]pyrene (1.0, 1.5, 2.0, 2.5, 3.0, and 4.0 µM) for 96 hrs, and cell viability was measured using the resazurin assay, also a real-time PCR and western blot techniques were employed to evaluate gene and protein expression respectively. Results: Viability assay demonstrated that the tested FICZ and BaP concentrations were no cytotoxic for the cellular system studied. When gene expression was evaluated, the final concentration of 100nM of FICZ proved to induced the AhR gene transcriptional activity promoting the increase of CYP1A1 mRNA levels ($p<0.05$) in each 24 hrs for 96 hrs of exposition. During this treatment, an unexpected decrease of AQP3 mRNA gene was also found during the FICZ 100 nM treatment compared to the control ($p<0.05$), specially at 72 and 96 hrs after exposition. It has been reported BaP, an important environmental pollutant and promoter of cellular oxidative stress, induced AQP3 mRNA over expression. This

was checked when exposed Hacat cells to 2.5µM of BaP (a pre-established concentration that induces the expression of AQP3) induced an increase in AQP3 mRNA expression, especially at 48 and 96 hrs after exposition in comparison to the control ($p<0.05$). Based on the opposite effects on AQP3, a co exposure of chemicals was evaluated at 96 hrs where both molecules presented a significant response. The co-treatment of 100 nM of FICZ ameliorated BaP AQP3 mRNA gene and of the protein levels indicated a possible competitive or antagonic mechanism between the two chemicals. Conclusion: The induction of an AQP3 mRNA downregulation stimulated by FICZ and the decrease in AQP3 protein levels in presence of BaP (an oxidative stress inductor) showed that FICZ could have a pivotal rol in the maintenance of skin health in presence of environmental pollutants.

Keywords: 6-formylindolo[3,2-b]carbazole, benzo[a]pyrene, AQP3

Introduction

The aryl hydrocarbon receptor (AhR) is a basic helix-loop-helix Per-Arnt-Sim (bHLH-PAS) family member that regulates many cellular processes like embryo development, cell cycle, apoptosis, proliferation and differentiation in many tissues like lungs, liver, heart and skin, where the receptor has an increased expression (Carlstedt-Duke, 1979; Okey, 2007; Tang et al., 2008). Once AhR is activated, dimerizes with its AhR translocator protein and travels to the nucleus, binds to xenobiotic response elements (XRE) inducing and regulating several genes (Li et

al., 2014; McMillan and Bradfield, 2007). Actually, exist many molecules that activate AhR, including toxic dioxins, polyphenols, polycyclic aromatic hydrocarbons (PAH) and tryptophan derived compounds (Nguyen and Bradfield, 2008). Emerging investigations suggest that environmental pollutants have a variety of detrimental effects on skin health (Wu et al., 2016). In this regard, it has been showing that chronic dermal exposure to PAHs may be related to skin disorders such as aging, pigmentary illness, acne, and cancer (Krutmann et al., 2014). Among all PAHs, benzo[a]pyrene (BaP) is recognized as a representative compound of that group, as well-documented carcinogenicity in humans has been established (Humans, 2012). However, the precise mechanisms by which BaP exposure harms the skin are still unclear.

FICZ (6-formylindolo[3,2-b]carbazole) is an UV-photoproduct of the aminoacid tryptophan (Wincent et al., 2008) that with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exhibits the highest AhR binding affinity (Tagliabue et al., 2019). Trough AhR, FICZ is able to induce the transcription of the XRE gene battery and its presence has been reported to increase severity in immune processes (rheumatoid arthritis and multiple sclerosis) and diminish severity of diseases like allergies, diabetes and skin disorders like psoriasis and atopic dermatitis (Rannug and Rannug, 2018). The mechanism of how FICZ diminished the severity of these disorders at skin cellular level are unknown. In the present study, we used a keratinocyte cellular model to evaluate the effects of FICZ on the expression levels of aquaporin 3 (AQP3), a small molecule transporter channel involved skin hydration, permeability, thickness and morphology of the tissue. Alterations in AQP3 function

has found in several skin treats like psoriasis, atopic eczema and vitiligo. (Blaydon and Kelsell, 2014; Hara-Chikuma et al., 2015; Kim and Lee, 2010; Olsson et al., 2006).

Material and methods

Chemicals

6-formylindolo[3,2-b]carbazole (FICZ) were purchased from Abcam (Cambridge, UK). Benzo[a]pyrene and resazurin sodium salt were purchased from Sigma-Aldrich (St Louis, MO,USA).

Cell culture

Human immortalized Hacat keratinocytes were cultured in Epilife medium culture (EDGS growth supplement; Life technologies, Carlsbad, CA) contained 100 U/ml penicillin and 100 µg/ml streptomycin in a 37 ºC in a humidified atmosphere of 5% CO2 and 95% air.

Viability assay with resazurin

Cell viability was investigate by seeding Hacat cells into 96 well plates at a cell density of 0.5x104/mL per well and cultured for 24 hours at 37 °C and 5% CO2 atmosphere. Subsequently, the medium culture was removed and the cells were washed once with phosphate-buffered saline solution (PBS) and replaced with

freshly EpiLife supplemented media with 1, 10, and 100 nM of FICZ or 1.0, 1.5, 2.0, 2.5, 3.0, and 4.0 µM of benzo[a]pyrene for 24, 48, 72 and 96 hours. At the finish of the treatment, the supernatant was discarded and replaced by freshly prepared resazurin reagent diluted in EpiLife supplemented medium to reach a final resazurin concentration of 30 µg/mL in a final volume of 100 µl per well. Plates were incubate immediately at 37 °C and 5% CO2 atmosphere for 2 hours. Fluorescent measurements with an excitation wavelength λ=560 nm and an emission wavelength λ=590 nm were performed to determine the degree of metabolism of resazurin in a Spectrofluorometer Synergy H1 (BioTek Instruments Inc. Winooski, VT, USA). Untreated Hacat cells were used as negative control, 0.5% of DMSO was used as vehicle control and cells treated with H2O2 (0.6%) were set as positive control.

FICZ and BaP cell exposition

In a reverse kinetic mode 3, 6, 9 and 12x104 Hacat cells/well were seeded in 24 well microplates. Nearly to 90% confluence, the cells were expose to 100 nM FICZ or 2.5µM benzo[a]pyrene, and incubated for 24, 48, 72 and 96 hours at 37 °C and 5% CO2 atmosphere. Because FICZ suffers a fast metabolism (Wincent et al., 2008), we prepared new FICZ in fresh medium and added it to cell monolayers each 24 hrs until finish the experiment in order to maintain the stimulus. Although benzo[a]pyrene not suffer the same fast metabolism (Bourgart et al., 2018), we maintained the same conditions for both chemicals, assuming a constant exposure. For co exposure experiments Hacat cells were seeded in 6 well microplates; upon reaching 90% of confluence, cell monolayers were co exposed to 100nM of FICZ and 2.5 µM of BaP

for 72 hrs at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. After treatment, Hacat cells monolayers were wash twice with PBS and after total RNA extraction was performed, gene expressions were evaluated.

Total mRNA isolation

After treatment with the chemicals, Hacat cell monolayers were wash twice with PBS1x and the RNAs were extract using TRIZOL® reagent (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA) following manufacturer´s instructions. Spectrophotometric analysis at 260 nm was perform on an Eon Microplate Spectrophotometer (BioTek) to assess final RNA concentration. The obtained samples were stored (-80°C) until their analysis.

Gene expression

Obtained RNA samples were subjected to reverse transcription according to manufacturer´s protocol, using Reverse Transcription Kit and OligodT primers (Applied BioSystems, Foster City, CA, USA), to obtain complementary DNA (cDNA). GAPDH, CYP1A1 and AQP3 were amplified by a real-time PCR detection system using specific TaqMan® probes (Applied BioSystems, Foster City, CA, USA), for each target gene using 100ng/µl of cDNA per sample. The thermal cycling process was established as follows: a preliminary denaturation step at 95 °C for 10 min, 40 cycles of PCR amplification at 95 °C (15 s), and 60 °C (60 s). The relative expression level of each candidate gene was individually normalize to the housekeeping gene

(GAPDH) and expressed as $2^{-\Delta\Delta Ct}$. The gene expressions were obtained in some cases, for each day.

Statistics

Prism software was used for statistical analyses 8.0 (GraphPad software 5.0, La Jolla, CA). The data were analyzed with the Shapiro-Wilk test to evaluate the normal distribution of the data. Data are present as mean ± standard error of the mean from at least three different experiments. When data were not normal, non-parametric (Friedman test, Kruskal Wallis) test was performed followed by Dunn´s post-hoc test to compare groups. Normal data were evaluated using t-student test. Values of $p \leq 0.05$ were considered significant.

Results

Cell viability

After exposure of Hacat cells to FICZ (1,10, and 100 nM) for 24, 48, 72 and 96 hours, resazurin were added to the media and cellular viability was measured according to materials and methods. Fig. 1a, shows that all the tested concentrations resulted no toxic for Hacat cells with the exception of 100 nM of FICZ and vehicle condition that showed reduced viability in the first 24 and 48 hrs that had a posterior recover by the end of the experiment. In agreement to ISO 10993-5, in “in vitro” cytotoxicity tests for biological devices or materials (International Organization for Standardization, 2009), which indicates that percentages above 80% are considered as no cytotoxic,

all the FICZ tested concentrations were harmless for our cellular system. For the follow experiments, we decided to use 100 nM of FICZ because is the most reported concentration of this photoproduct in several cellular lines and the one that has shown important biological effects. BaP viability assay showed low toxicity at any used concentration in the Hacat cell system compared to control (data not shown).

CYP1A1 and AQP3 gene expression by FICZ

As well as TCDD, FICZ has a high affinity for AhR receptor and induce the AhR DNA binding to specific promoter regions in order to modulate diverse gene expression (Tagliabue et al., 2019). CYP1A1 is the most representative gene of AhR pathway and the induction of gene expression is tracking by its upregulation. We evaluated the expression of CYP1A1 each 24 hrs for 96 hrs in continual exposure to 100 nM FICZ (Fig. 2 a-d), and found that in each evaluated time of exposition CYP1A1 expression increased in a significant manner compared with the control ($p<0.05$), with the exception of 72 hrs. Despite no significate difference were found in this condition, a tendency to increase was registered. Because FICZ is an UVB tryptophan photoproduct that has been found in human skin extracts, we decided to evaluate if FICZ could modulate AQP3 gene expression, which deregulation has been described in several skin diseases like atopic dermatitis, vitiligo, psoriasis and even skin cancer induced by chemicals (Hara-Chikima and Verkman, 2008). Fig. 3 a-d, shows the expression levels of AQP3 for each 24 hrs. for 96 hrs. in continual exposure to 100 nM FICZ replaced each 24 hrs. At 24 and 48 hrs. of exposure showed no difference compared with the control but after 72 hrs and 96 hrs of

treatment the AQP3 gene expression was downregulated in a significant manner ($p<0.01$ and $p<0.05$, respectively) compared to the control and vehicle.

AQP3 gene expression of benzo(a)pyrene

Benzo(a)pyrene (BaP) is a polycyclic aromatic hydrocarbon related to mutagenesis and carcinogenesis in humans and other animal models via induction of oxidative stress (Burchiel et al., 2007; Colapietro et al., 1993; Costa et al., 2010; Vázquez-Gómez et al., 2018). It has been suggested that BaP could promote toxicity in skin tissue since BaP induce AQP3 gene expression (Bui et al., 2016). Because 2.5µM BaP has shown upregulate AQP3 expression levels in the study of Bui, et al (2016), we used this concentration in our cellular system to induce AQP3 expression. Fig 4 a-d shows AQP3 mRNA expression during 96 hrs of exposition to 2.5µM BaP. It is notorious that during the first 24 hrs AQP3 mRNA increased in presence of the chemical, but no in a significant way at difference of 48 hrs of exposition where the gene expression increased two fold times compared with the control ($p<0.05$). At 72 hrs were not found any difference with the control, contrary to the 96 hrs of treatment, were AQP3 mRNA increased was evidenced ($p<0.05$).

AQP3 expression in co exposure with FICZ and BaP

Based on the relevance of AQP3 in skin homeostasis, cancer development and the opposite effects shown on its mRNA levels in presence of FICZ and BaP, a co exposure of chemicals was evaluated at 96 hrs, an exposition time where both

molecules presented a significant response. Hacat cells were co exposed to 100 nM of FICZ and 2.5µM of BaP and the AQP3 mRNA expression was reevaluated. Fig. 5 shows that 100 nM of FICZ and 2.5µM of BaP induced a significate downregulation and an upregulation respectively compared to the control ($p<0.05$). When we co treated the cells with 100 nM of FICZ and 2.5µM of BaP a decrease in AQP3 mRNA expression was observed, founding no significant differences against the control but with AQP3 mRNA expression reported to BaP alone ($p<0.05$). It seems that the co-treatment of FICZ ameliorated BaP AQP3 mRNA gene and of the protein levels indicated a possible competitive or antagonic mechanism between the two chemicals.

Discussion

FICZ is a tryptophan photoproduct produced in the skin by several environmental and biological conditions like ultraviolet radiation, visible light, the presence of Malassezia yeast species in the tissue and metabolic inner reactions in the body where tryptamine, indol-3-piruvate, H_2O_2 and even enzymes like the aspartate aminotransferase, give birth to this molecule (Smirnova et al., 2016). The activity of FICZ has been well documented in clinical skin treats like psoriasis and atopic eczema where inflammatory reactions and genes involved in the disease were down regulated (Rannug and Rannug, 2018). Many of the effects of FICZ are mediated by the aryl hydrocarbon receptor (AhR), a cytosolic protein of high prevalence in the skin tissue that controls many cellular processes such as cell cycle arrest, proliferation, differentiation and apoptosis (Larigot et al., 2018). Some researchers

have considered FICZ as the physiological ligand of AhR by its efficient production and metabolism, its high affinity for its receptor and the most important, the induction of AhR gene battery. FICZ induce its own metabolism promoting CYP1A1 activation and expression. In our study, we showed that 100nM FICZ induced the upregulation of CYP1A1 mRNA expression at 24, 48, 72 and 96 hrs of treatment (Fig 2a-d). In the skin, AhR controls the gene expression of several proteins like aquaporin 3 (AQP3), a water and glycerol transport protein involved in proliferation, migration, maintenance of skin hydration, elasticity, and wound healing of keratinocytes in the epidermis (Blaydon and Kelsell, 2014). A study performed by Hara-Chikuma and Verkman reported AQP3 overexpression plays a critical rol in skin cancer development chemically induced in murine models (Hara-Chikuma and Verkman, 2008). Several chemicals like TCDD (2,3,7,8-Tetrachlorodibenzodioxin), a carcinogenic dioxin and benzo(a)pyrene, a tumorigenic and carcinogenic polycyclic aromatic hydrocarbon, have shown to induced the gene levels of the protein channel (Linh-Chi, et al., 2016). In the present study, AQP3 mRNA expression was downregulated in Hacat cells exposed to 100 nM FICZ (Fig 3a-d). Similar results were found in psoriasis and vitiligo where absence of AQP3 was related to skin dehydration, differentiation problems and reduced keratinocyte survival (Kim and Lee, 2010; Lee et al., 2012). Downregulation of AQP3 expression by other chemicals is not usual, or at least not frequently documented. In this regard, neonatal normal human epidermal keratinocytes (NHEKS) treated with 40 µM of resveratrol (an AhR antagonist) “in vitro” diminished AQP3 gene expression (Wu et al., 2014). As we mentioned, deregulation of AQP3 expression is present in several skin clinic treats included cancer where the elevated presence of reactive oxygen species (ROS) and

therefore whit oxidative stress has shown played a significant role in its development. Benzo[a]pyrene (BaP) is the most widely compound used for studying the oxidative stress toxic effects of polycyclic aromatic hydrocarbons in the skin cells (Costa et al., 2010; Melchini et al., 2011; Padmavathi et al., 2005; Yusha et al., 2018). Despite evidence suggest AQP3 gene transcription could be induced by BaP, there is no studies at this respect in skin models, only in human hepatocytes (HepG2 cell line) where BaP upregulated AQP3 mRNA expression (Linh-Chi et al., 2016). In our study, we found BaP induced an increase in AQP3 mRNA levels in the majority of our experimental conditions (Fig 4a-d). Similar results were reported by Hieda et al (2020) who found AQP3 upregulate its mRNA expression levels as a result of ROS increment in a human epidermis model exposed to particle matter (Hieda et al., 2020).

Based on the relevance of AQP3 in skin homeostasis, cancer development and the opposite effects shown on its mRNA levels in presence of FICZ and BaP, a co exposure of chemicals was evaluated in our study at 96 hrs where both molecules presented a significant response. We found that AQP3 mRNA levels in co treatment of 100 nM of FICZ and 2.5 µM of BaP diminished (Fig. 5) in comparison to BaP treatment, showing no significant difference with the control. This indicate a possible competitive or antagonic mechanism between the two chemicals. In relation to this, a study by Gan et al (2020) showed that 10 nM FICZ in co treatment with 1µM BaP diminished ROS production and % of apoptotic cells in a mitochondrial dependent mechanism in the murine hepatoma cell line Hepa1-6 (Gan et al., 2020).

The regulatory effects on AQP3 protein expression showed in this study could also be related to the differential affinity by the AhR receptor between the molecules: FICZ induced a half-maximal response of AhR at 1nM meanwhile BaP does at 617 nM (Tagliabue et al., 2019). This explains that FICZ first induced a fast activation of AhR and downregulates mRNA AQP3 levels, an effect that cannot be reversed by BaP, a molecule whit less affinity and in turn, uncapable to upregulates AQP3 gene expression. Special attention in this decrement of AQP3 gene expression is important because if FICZ could regulate the AQP3 behavior protein in a positive grade to ameliorate skin disorders (including cancer), it will be a promising use of this molecule in these pathologies. One probe of the beneficial effects of FICZ were reported by Murai et al (2018) who found the photoproduct inhibited several metalloproteinases, avoiding the collagen over production in normal dermal fibroblast in a MEK and ERK dependent-manner. This effect could be useful to treat scleroderma and fibrotic skin disorders (Murai et al., 2018). The induction of an AQP3 mRNA downregulation stimulated by FICZ and the decrease in AQP3 protein levels in presence of BaP (an oxidative stress inductor) showed that FICZ could have a pivotal rol in the maintenance of skin health in presence of environmental pollutants.

Conclusion

The skin is a dynamic tissue in constant renovation. Its exposition to environmental factors like sunlight, microbiota colonization or natural metabolic reactions in the human body, could induce the synthesis of FICZ, a tryptophan metabolite that

activates AhR. The results presented herein suggest that AQP3, a protein altered in several skin pathologies including cancer, is downregulated in Hacat cells exposed to FICZ. In our knowledge, this is the first study that show this AQP3 behavior in human keratinocytes. Nonetheless, the nature of this decrease was not clarified; therefore, further investigations are required to understand the influence of results found in this investigation on skin cells exposed to environmental pollutants as BaP.

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Declaration of interest

The authors declare no conflict of interest

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Figure legends

Fig. 1 Cell viability assay.

Increasing concentrations of FICZ were used. Resazurin assay was performed 24, 48, 72 and 96 hrs of exposure. Percentage of viable cells were calculated and expressed as the average of three independent experiments, each condition in quadruplicate.

Fig. 2 FICZ CYP1A1 gene expression profile

Hacat cells were exposed to FICZ 100 nM for 96 hrs. Each day of treatment, new FICZ were added to fresh media in order to maintain the stimulus. a-e shows CYP1A1 gene expression at 24, 48, 72 and 96 hrs respectively; GAPDH was used as a house-keeping gene; *P<0.05, versus untreated controls. The results are representative of three independent experiments.

Fig. 3 FICZ AQP3 gene expression profile

Hacat cells were exposed to FICZ 100 nM for 96 hrs. Each 24 hrs of treatment, new FICZ were added to fresh media in order to maintain the stimulus. a-e shows AQP3 gene expression at 24, 48, 72 and 96 hrs respectively; GAPDH was used as a house-keeping gene; *P<0.05 and **P<0.01 versus untreated controls. The results are representative of three independent experiments.

Fig. 4 BaP AQP3 gene expression profile

Hacat cells were exposed BaP 2.5µM for 96 hrs. Each 24 hrs of treatment, new BaP were added to fresh media. a-e shows CYP1A1 gene expression at 24, 48, 72 and 96 hrs respectively; GAPDH was used as a house-keeping gene; *P<0.05, and ***P<0.001, versus untreated controls. The results are representative of three independent experiments.

Fig. 5 AQP3 expression in co exposure with FICZ and BaP

Hacat cells were exposed to 100 nM of FICZ, 2.5µM of BaP or a combination of both chemicals for 96 hrs and AQP3 mRNA levels were evaluated. GAPDH was used as a house-keeping gene; *P<0.05, versus untreated controls. The results are representative of three independent experiments.

Figures

Figure 1

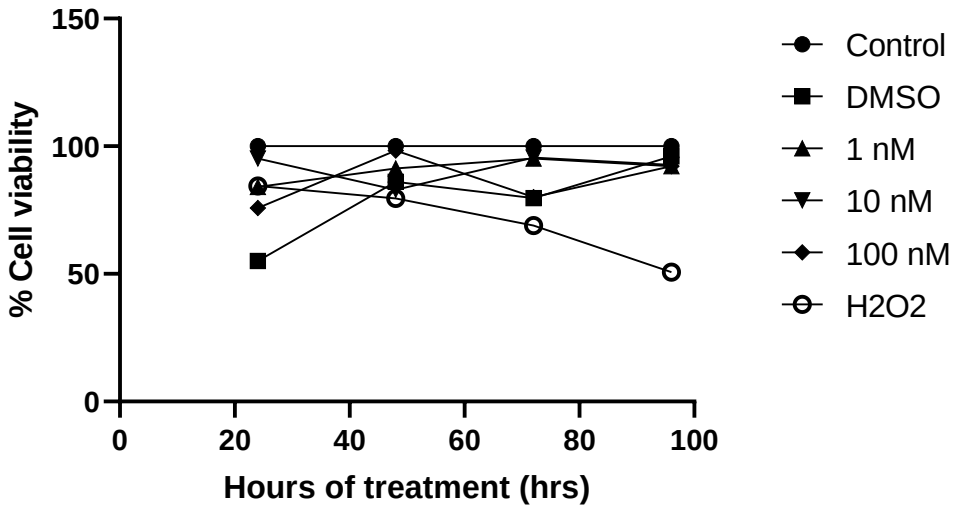
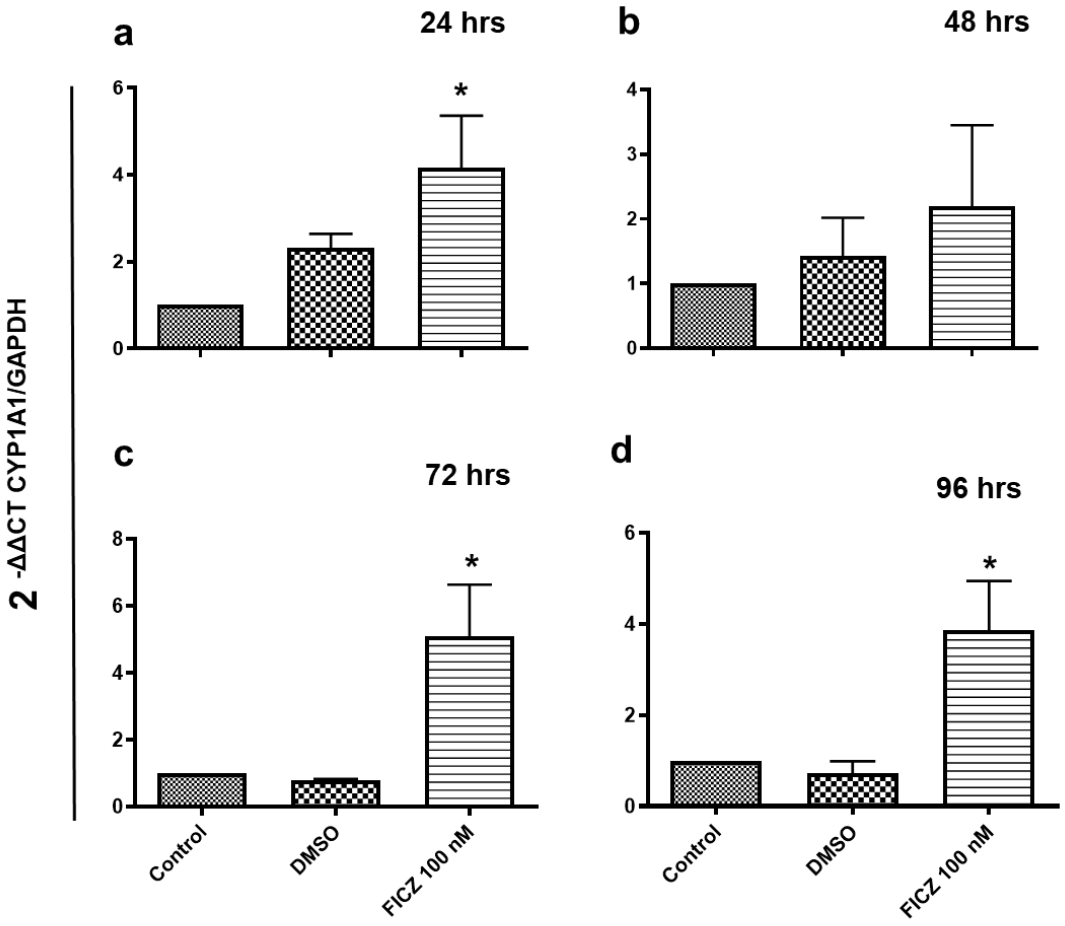


Figure 2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24 hrs
b
48 hrs
c
72 hrs
d
96 hrs
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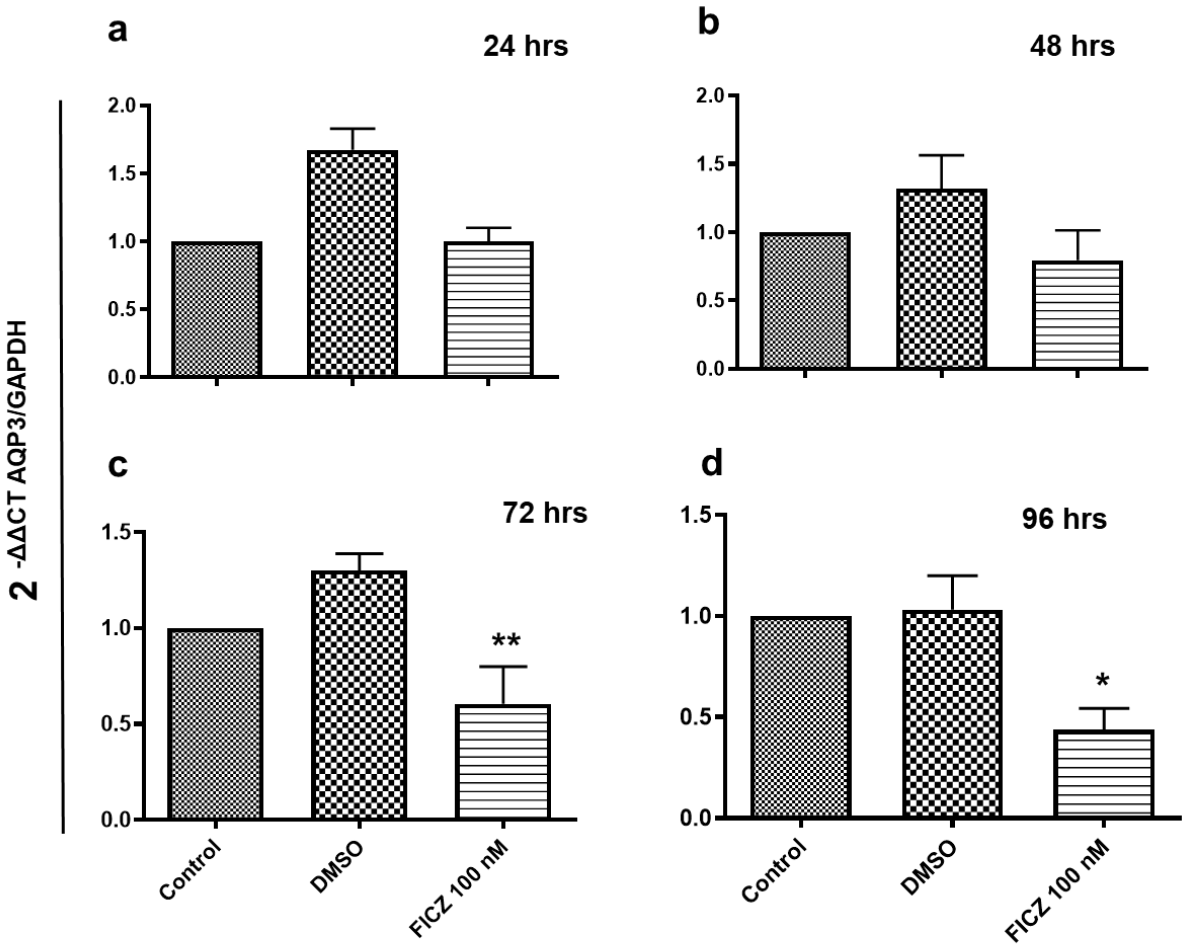
*
Control
DMSO
FICZ 100 nM

Figure 3


a
24 hrs
b
48 hrs
c
72 hrs
d
96 hrs
2 -ΔΔCT AQP3/GAPDH
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1.5
1.0
0.5
0.0
**
*
Control
DMSO
FICZ 100 nM

Figure 4

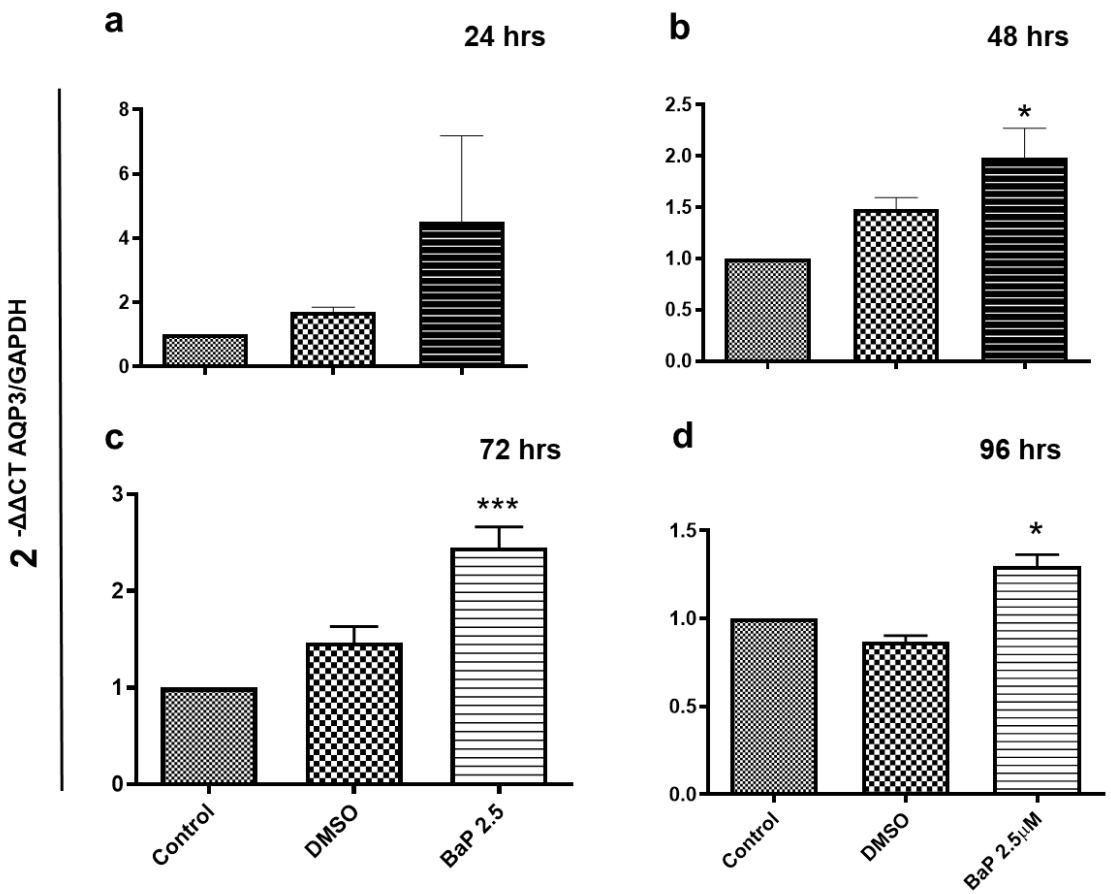
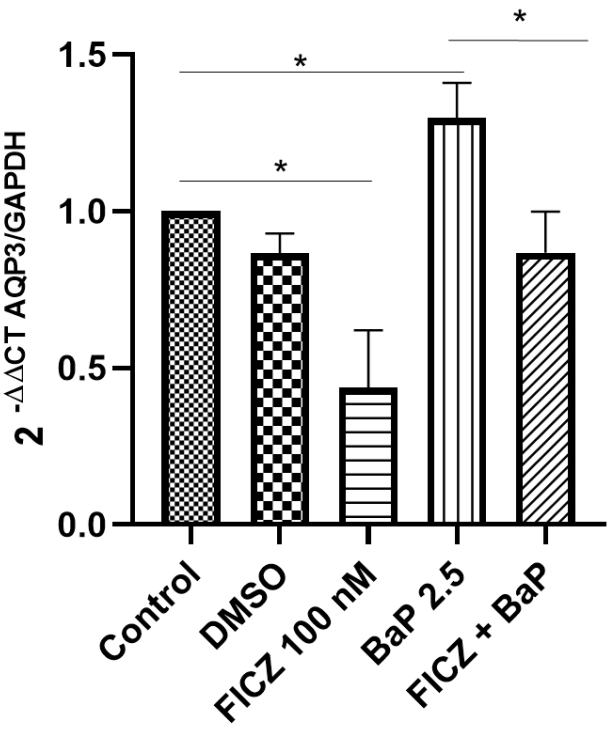

a
24 hrs
b
48 hrs
c
72 hrs
d
96 hrs
2 -ΔΔCT AQP3/GAPDH
*
***
*
Control
DMSO
BaP 2.5
Control
DMSO
BaP 2.5μM

Figure 5


2 -ΔΔCT AQP3/GAPDH
1.5
1.0
0.5
0.0
*
*
*
Control
DMSO
FICZ 100 nM
BaP 2.5
FICZ + BaP