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**Temporal transcriptomic analysis of human primary keratinocytes exposed to  $\beta$ -naphthoflavone highlights the protective efficacy of skin to environmental pollutants.**

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## **Abstract**

The skin covers almost the entire body and plays an important role in detoxification and elimination of xenobiotics. These processes are initiated following the binding of xenobiotics to the aryl hydrocarbon receptor (AhR), which leads to the expression of several detoxification enzymes. To gain some insights on their impacts on skin cells over time, a temporal transcriptional analysis using gene expression arrays was performed in human primary epidermal keratinocyte (HEK) cells exposed for 6, 24 and 48h to  $\beta$ -naphthoflavone ( $\beta$ NF), a potent agonist of AhR. Our results demonstrated that expression of genes related to xenobiotic, inflammation, and extracellular matrix remodeling was increased upon  $\beta$ NF treatment from 6h onwards. In contrast, the anti-oxidative response was seen mainly starting at 24h. While some of the genes controlled by the epidermal differentiation complex was induced as soon as 6h, expression of most of the S100 related genes located within the same chromosomal locus and keratin genes was increased at later times (24 and 48h). Altogether our transcriptomic data highlight that following  $\beta$ NF exposure, HEK cells elicited a protective xenobiotic response together with the activation of inflammation and keratinocyte regeneration. Later on these processes were followed by the stimulation of anti-oxidant activity and terminal differentiation.

Keywords : Xenobiotics,  $\beta$ -naphthoflavone, protective response, keratinocyte

## **Introduction**

The skin is a full part organ with an area ranging from 1.5 to 2 m<sup>2</sup> for an adult human being representing about 15% of the total weight. This tissue consists of three layers: the epidermis, the dermis and the hypodermis. Within the epidermis, keratinocytes interact with various chemicals, either by absorption after topical exposure, known as the cutaneous route, or by the vasculature that feeds it from the dermis, named the

systemic route. The outermost layer, the stratum corneum (SC), is considered as the major barrier against skin penetration of chemicals by the topical route (Trommer and Neubert, 2006). Besides being a simple physical barrier, as a sensitive organ, the skin also exhibits more active functions involved not only in peripheral but also in systemic homeostasis (Slominski et al., 2018, 2012). Skin cells are able to use classical stress neurotransmitters, neuropeptides, and hormones to enhanced protection function. This can be stimulated by different factors as ultraviolet radiation (UVR), biological factors (infectious and noninfectious), and other physical and chemical agents.

It has also been known for decades that skin expresses an active enzymatic system (Oesch et al., 2014a; Pannatier et al., 1978) capable of interacting with absorbed xenobiotics and facilitating their elimination from the body. Two phases of metabolism called phase I and phase II reactions (Rushmore and Tony Kong, 2002) are distinguished according to the transformation processes induced by the xenobiotic metabolism enzymes (XME). Most liver detoxification enzymes are present in the skin and localized in the epidermis (Baron et al., 2001; Hewitt et al., 2013; Lepoittevin, 2004; Oesch et al., 2014a).

While phase I metabolism is associated with the formation of reactive and potentially toxic derivatives, phase II metabolism reduces the metabolite reactivity by conjugation. In the course of this process, among the derived metabolites, a more reactive and possibly toxic form than the parent molecule can be generated. It is now accepted that cutaneous metabolism can contribute to the initiation of pathological events. This mainly concerns the activation of small, absorbable molecules in the skin that can trigger a sensitization reaction, the first step in allergic contact dermatitis (Smith Pease et al., 2003). Other toxic effects such as genotoxicity (Brinkmann et al., 2013) or induction of carcinogenesis (Wiebel and Gelboin, 2010) may be triggered by only few

cutaneous metabolites in contrast to the high number of those involved in sensitization-related problems.

Mechanisms that are involved in oxidation and / or reduction reactions of different substrates depend mainly on the enzymes of the CYP family. Although the presence of CYP messenger RNA in healthy and untreated human skin has been demonstrated, some authors have observed that these enzymes are not active in the skin (van Eijl et al., 2012). However, other studies have reported that enzymatic activities triggered by distinct CYPs (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2E1, CYP2D6, CYP3A4/5) can contribute to the metabolism of procarcinogens and drugs in skin (Ahmad and Mukhtar, 2004). The CYP1A family, known to be strongly induced in response to polycyclic aromatic hydrocarbons (PAH) in the liver, is used as a biomarker of exposure to environmental pollutants (Baron and Merk, 2001). The ability of the skin to respond to PAH exposure in skin models has been studied, and these models were validated based on the expression and inducibility of CYP enzymes (Neis et al., 2010). However, the impact of this modulation has never been correlated with a change in the skin bioavailability of a substance absorbed into the skin.

Xenobiotics, as nuclear receptor ligands, can bind to transcription factors leading to modulate the expression of genes encoding xenobiotic metabolizing enzymes (XMEs). Once XME mRNAs are synthesized, it takes 24 to 48 hours to detect their corresponding protein products. Production of these enzymes increases the metabolism of xenobiotics and depending on the cytotoxicity of the resulting metabolites, deleterious processes can arise (Shimada and Fujii-Kuriyama, 2004). Thus, the control of these processes as well as their impacts should be tightly regulated by cellular specific mechanisms. While not all enzymes are inducible, drugs such as phenobarbital or omeprazole, ethanol from alcohol consumption or combustion products, such as

PAH, found in cigarette smoke or in exhaust gas, have an ability to induce XME genes (Le Vee et al., 2010; Oesch et al., 2014b; Shimada and Fujii-Kuriyama, 2004). Guéguen *et al.* (Guéguen et al., 2006) have listed the transcription factors involved in the induction of xenobiotic metabolism genes in humans and specified examples of agonists. The most known and characterized transcription factors are the receptors for pregnane (PXR), polyunsaturated fatty acids (PPAR), androsterone (CAR) and aromatic hydrocarbons (AhR). Various mechanisms can lead to activation of transcription. Internalized xenobiotics in the cell can directly activate the receptors in the cytoplasm before being translocated into the nucleus such as for CAR following its phosphorylation induced by the xenobiotic. PXR and PPAR are localized in the nucleus and bind to xenobiotic via their hydrophobic pocket. The resulting complex interacts to another essential player, RXR (retinoic acid receptor) to activate the transcription of detoxification genes (Kliwer et al., 2002). CAR's activity is also dependent of RXR (Xu et al., 2005). Activation of the AhR receptor bound to a xenobiotic requires, through its interaction with Arnt (AhR nuclear translocator), to be free from its interactors, several chaperone proteins located in the cytoplasm under basal conditions. The Arnt/AhR will then bind to the XRE (Xenobiotic Response Element) promoter motif to activate gene transcription. Even though these mechanisms have been identified mainly in the liver, induction of some of their gene targets have been detected in the skin (Pavek and Dvorak, 2008). Activation of the AhR receptor, involving PAH, has been demonstrated in the induction of CYP in human keratinocytes (Swanson, 2004). Transcripts of CYP 1A1 and 1A2 were also induced in explants of adult human skin after exposure to hard coal, a major source of PAH (Smith et al., 2006).

Herein, we wanted to investigate, at a temporal transcriptomic level, the sequential steps leading to the ultimate impacts of xenobiotics on skin cells. Human epidermal

keratinocytes (HEK) were exposed either for 6, 24 or 48h to  $\beta$ -naphthoflavone ( $\beta$ NF), which is often used as an AhR receptor agonist and a CYP inducer (Patel et al., 2007).  $\beta$ NF is a substance of synthetic origin which belongs to the family of polycyclic aromatic hydrocarbons, also known as a putative chemo-preventive agent (Izzotti et al., 2005). At first, induction of the known main effectors was confirmed at RNA levels by RT-qPCR and measurements of their enzymatic activities. Transcriptomic data were then analyzed by PredictSearch<sup>®</sup> software, which allowed to identify functional networks highlighting the temporal effects triggered by  $\beta$ NF on keratinocytes such as HEK.

## **Materials and method**

### **Cell culture and treatment**

Neonatal human epidermal keratinocytes (HEKn) are primary cells derived from neonatal foreskins. They are supplied by the American Type Culture Collection (ATCC) in the form of  $5 \times 10^5$  viable cells vials from different donors in order to have a constant homogeneity of cell populations. These cells were cultured in a specific medium (Epilife, Life Technologies) supplemented with a plant-derived supplement (HKSdaFREE and HKGE, AvantBio) that replaces the sera of animal origin more commonly used in cell culture. The cells were cultured at 37°C in an incubator (5% CO<sub>2</sub> / 95% air). Routinely, the cells were grown in 225 cm<sup>2</sup> flasks and their culture medium was changed every 2/3 days. These are adherent cells that attach spontaneously to the plastic on which they are seeded without the need to add matrix. For metabolism induction studies, cells were cultured with the same culture medium in 12 well plates for enzymatic activity measurements or in 75 cm<sup>2</sup> flasks for PCR and transcriptomic studies. Early passages (between the third and fifth passage) of the cells were used to

obtain consistent results among the different experimental conditions.  $\beta$ NF being poorly water-soluble, stock solutions in a compatible solvent had to be prepared. Dimethyl sulfoxide (DMSO, Sigma-Aldrich) was used to prepare intermediate solutions before final dilution in the culture medium. Its final concentration did not exceed 0.2% in order to avoid cytotoxicity. At subconfluence, HEK were treated with 10 $\mu$ M  $\beta$ NF (CAS no. 6051-87-2, Sigma-Aldrich) diluted in culture medium for the indicated times.

### **Cytotoxicity assessment**

Cell viability were performed to ensure that the inducers tested did not interfere with cell survival. Cells were incubated for 3 hours with a MTT (Methylthiazolyldiphenyl tetrazolium, Sigma-Aldrich) solution diluted in culture medium. After incubation, the MTT solution was aspirated and replaced with isopropanol to solubilize the crystals resulting from the MTT reduction by the mitochondrial enzymes of living cells. Absorbance was measured spectrophotometrically at 570 nm and is proportional to the number of living cells. To calculate cell viability, the absorbance of an untreated control condition, was compared to the absorbance of a treated condition.

### **Quantitative Real-Time PCR**

Total RNAs were extracted from HEK cells using RNeasy Mini Plus Kits (Qiagen) and reverse transcribed using the High-Capacity RNA-to-cDNA kit (Applied Biosystem) according to the manufacturer's instructions.

Real-time PCR (qPCR) was performed using the primers (see Table 1) and PowerUp SYBR Green Master Mix (Thermofisher).

Products were amplified with a 7500 Real Time PCR System (Applied Biosystem) using the following program: 10 min at 95°C, followed by 40 cycles of 15 sec of denaturation at 95°C, 1 min of hybridization / elongation at 60°C. Samples were analyzed using the  $\Delta\Delta$ Ct method. Briefly, Ct values for the housekeeping gene,



GAPDH, were subtracted from Ct values of the gene of interest ( $\Delta Ct$ ). The  $\Delta Ct$  values for untreated controls were subtracted from the  $\Delta Ct$  for the treated samples ( $\Delta\Delta Ct$ ). Messenger RNA (mRNA) fold change was determined using  $2^{\Delta\Delta Ct}$  according to the ratio between treated versus untreated conditions. Sequences of the primers for the genes tested are provided in the table below.

Target genes	Forward primers (5'→3')	Reverse primers (5'→3')
<b>CYP3A4</b>	CTTCATCCAATGGACTGCATAAAT	TCCCAAGTATAACACTCTACACAGACAA
<b>CYP1A1</b>	GCACAGAGGTAGTCTCACTGCTTG	AAGGGCAGAGGAATGTGATGTT
<b>CYP1A2</b>	TGGAGACCTTCCGACACTCCT	CGTTGTGTCCCTTGTGTGC
<b>CYP2B6</b>	TTCCTACTGCTTCCGTCTATCAAA	GTGCAGAATCCCACAGCTCA
<b>GAPDH</b>	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG

### Microarray assay

Following qPCR pre-validation, the RNA samples (one sample per condition) were used for amplification, labeling and hybridization with the Agilent one-color microarray protocol (Low Input Quick Amp Labeling One Color Kit) (Agilent Technologies). Briefly, total RNAs were reverse-transcribed into cDNA using the T7 promoter primer. Cyanine-3-labeled cRNAs from cDNA were synthesized in a solution containing dNTP mix, T7 RNA polymerase and cyanine 3-dCTP that was incubated at 40°C for 2 hours. Labeled cRNAs were purified and fragmented through an incubation at 65°C for 17 hours before hybridization on Agilent 8×60 K (AMADID 039494, Agilent Technologies) containing 62 975 oligonucleotide probes. Raw microarray signals were scanned and extracted using Agilent Feature Extraction Software 11.0.1 (Agilent Technologies). Each chip image (.tif) was verified and validated according to quality control reports (QCreports) from the software to evaluate the quality and reproducibility of the hybridizations. Following image analysis, the Feature Extraction software and the limma R packages in Bioconductor were used for further pre-processing. The

Bioconductor package has function for reading the data, plotting images, and for normalizations within and between arrays (Quantile methods). The limma package also implements linear modeling for selecting differentially expressed genes and has functions for alternative methods of background correction. During data quantification by Feature Extraction software the background values are provided by QCReports and the values of GlsWellaboveBG are indicated by either 0 (lower than background) or 1 (higher than background). In our study only the values equal to 1 are selected for treated conditions. Among these values only intensities values higher than two times the background intensity are kept for further analysis. These data were deposited in the public domain after GEO Submission and are available under the accession number: GSE143748.

### **Microarray data analysis**

Following normalization, only genes with distinct relative background intensities ( $\text{intensity} \geq 50$ ) were selected. From the intensities obtained, the induction ratios ( $R_i$ ) of the treated samples compared to the untreated controls were calculated for each gene and each treatment condition. A selection of modulated genes in response to the inductors was then made by establishing induction and repression intensity ratio thresholds of 1.5 and 0.6, respectively. Selected genes were submitted to PredictSearch<sup>®</sup> software (Laboratoire Genex). This text mining-based software characterizes the pathways and functional networks in which the selected genes are involved through the identifications of functional correlations between genes and terms co-cited in PubMed abstracts.

The relevance of functional correlations was validated by a Fisher test that allowed a statistical analysis of the gene/term co-citations.

### **EROD enzymatic activities measurement**

The activities of CYP1A1 / 2 and CYP2B were determined using the 7-ethoxyresorufin (7ER) substrate specific for these isoforms purchased from Sigma-Aldrich. 7ER is converted to resorufin (RESO) by 7-ethoxyresorufin O-deethylation (EROD), in presence of the NADPH cofactor. The experiment was carried out directly on living cells, *in situ*, by replacing the usual culture medium with the same medium supplemented with 7-ethoxyresorufin at 1  $\mu$ M. Dicoumarol (Sigma-Aldrich) at 2 mM was also added to the medium as a phase II enzyme inhibitor to prevent the product of the EROD reaction to be degraded. After one hour of incubation in the oven, samples of 200  $\mu$ l of culture medium were removed and deposited in a 96-well black plate. Fluorescence was then measured by the Fluostar machine at the following wavelengths: 560 nm excitation wavelength and 590 nm emission wavelength (Radenac et al., 2004). The result of the fluorescence measurement is expressed in relative fluorescence units (U.R.F). To determine the amount of resorufin formed in each sample, a calibration line was generated from resorufin standards (Sigma-Aldrich) at known concentrations (7 serial dilutions to half from 250 to 1.95 nM).

### **Statistical Analysis**

The data are presented as means  $\pm$  SD (standard deviation). Statistical differences in the data were evaluated by Student's t-test or one-way ANOVA as appropriate and were considered significant at  $p < 0.05$ .

### **Results and discussion**

#### *Evaluation of the viability of HEK293 cell after $\beta$ -naphthoflavone treatments*

$\beta$ NF is a well-known characterized CYP inducer (Boobis et al., 1977; Oesch et al., 2014). In order to overcome biases induced by cytotoxic effects following exposure to

$\beta$ NF, we have defined the experimental conditions which preserve the survival of cell cultures. Viability results from MTT assay are shown in table 1. After 14h of exposure, a cytotoxic effect was detected between 10 and 100  $\mu$ M while after 24h or 48h, cytotoxicity was observed for doses between 5 and 10  $\mu$ M. These ranges confirmed what was reported in the literature and allowed to choose 10  $\mu$ M as the dose to be used for further experiments.

#### *Evaluation of CYP expression by RT-qPCR in response to $\beta$ NF in HEK cells*

At first, we wanted to evaluate the efficiency of our treatment with  $\beta$ NF in human primary keratinocytes (HEK) as well as the quality of our RNA extraction before performing gene expression profiling. To do so, we chose to quantify by RT-qPCR the induced expression of genes encoding cytochrome P450 enzymes at 48h, such as *CYP1A1*, *CYP1A2*, *CYP3A4* and *CYP2B6* (Table 2). Among the different members of the same family, *CYP3A4* is involved in the metabolism of a high number of therapeutical drugs used currently and *CYP2B6* metabolizes anti-cancer drugs such as cyclophosphamide and ifosphamide (Oesch et al., 2014b). As shown in table 2, at 48h, CTs in treated conditions were significantly lower than in untreated conditions for *CYP1A1* and *CYP1A2*. Accordingly, fold changes calculated from  $\Delta\Delta$ CTs deduced from the ratio between treated versus untreated conditions after normalization with GAPDH (see material & methods) demonstrated that  $\beta$ NF was able to induce efficiently these genes in HEK. In contrast, expression of *CYP3A4* or *CYP2B6* was not detected in these cells even in presence of  $\beta$ NF although *CYP3A4* expression was reported to be stimulated upon dexamethasone treatment of human keratinocytes (Baron and Merk, 2001). Thus, we confirmed that  $\beta$ NF is able to stimulate the transcription of specific

cytochrome P450 genes such as *CYP1A1*, and *CYP1A2* in human cultures of keratinocytes.

#### *CYP1A1 activity measurement through EROD assessment*

To investigate whether change in gene expression and protein levels of CYP1A1 was also correlated with the activity of the encoded enzyme, CYP1A1 enzymatic measurements towards an ethoxyresorufin-O-deethylase (EROD) assay (Burke and Mayer, 1974; Sulaiman et al., 1991) were performed. HEK cells were either mock-treated or treated with  $\beta$ NF at different concentrations for 48 and 72h. Maximal activity as deduced from the fold change calculated between treated and untreated cell extracts was detected at 0.5  $\mu$ M and 1  $\mu$ M after 72h treatment (Fig. 1). However higher concentrations (5 and 10  $\mu$ M) elicited a lower activity, likely due to an inhibition by excess of substrate, and such effects were also reported in hepatocytes (Lemaire et al., 2011; Petrusis and Bunce, 1999). As illustrated by the strong induction of CYP1A1 mRNA and the corresponding enzymatic activity, we concluded that our experimental conditions were efficient to initiate a signaling triggered by AHR in response to  $\beta$ NF.

#### *Temporal gene expression profile of $\beta$ NF-treated HEK*

The efficient induction of *CYP1A1* prompted us to investigate in more details the temporal transcriptomic response of HEK upon  $\beta$ NF treatment. Thus, a whole human gene expression profiling was performed using total RNAs extracted from either mock treated cells or  $\beta$ NF-treated HEK cells after 6, 24 and 48h of treatment.

After intra-chip and chipset normalization of the raw data, only genes with intensities  $\geq$  50 (significantly distinct from the background) were retained. This filtering selects 24,375 gene sequences. Fold changes (Fc) were calculated from the ratio between intensity values of the  $\beta$ NF-treated samples versus those of the untreated samples. The

modulated genes were selected for an  $F_c$  greater than or equal to 1.5 for genes induced by  $\beta$ NF or less than or equal to 0.6 for genes repressed by  $\beta$ NF. Although only one sample was used by condition, genes were selected at first when their expression was changed with a same tendency at least at two consecutive time points. As seen in Table 3, a significant number of genes remained induced at the 3 kinetic time points. However the transcriptional effect of  $\beta$ NF on HEK cells seemed to be more activator than repressor and somehow was delayed since the largest number of modulated genes was detected between 24 and 48h.

Furthermore, the selected genes were submitted to PredictSearch<sup>®</sup> software to identify the correlations based on the co-occurrence of terms and genes in the scientific literature allowing to integrate them into functional networks (Burke and Mayer, 1974; Sulaiman et al., 1991). These functional networks were then eventually completed with genes whose expression was modulated at individual time points (6, 24 or 48h) but in this case with a  $F_c \geq 2$  or a  $F_c \leq 0.5$ .

Supporting our RT-qPCR data, while *CYP1A1* and *CYP1A2* expression was detected at each time point, a strong maximal induction was seen for both genes at 48h (Table 4). For *CYP1B1*, the peak was reached at 24h. As illustrated in Figure 2, expression of these genes are triggered after the activation of AhR/ARNT signaling pathway leading to the stimulation of the xenobiotic response element (XRE) in response to  $\beta$ NF (Walsh et al., 2013; Siddens et al., 2015; Zhu, 2010). Moreover, several other CYP genes (*CYP27A1*, *CYP27B1* and *CYP5A1*) were induced. Among them, it has been reported that the product encoded by *CYP5A* plays important roles in cytochrome P450-mediated drug metabolism (Zhu, 2010). In addition to *CYP5A*, an induced expression of *CYP5R2*, cytochrome b5 reductase 2, was similarly induced at 24 and 48h (Table 4).

Noteworthy, expression of *AhR* itself was repressed by  $\beta$ NF at 48h suggesting the initiation of a negative feedback loop to slow down the XRE response. However, a gene encoding a potential AhR interactor, ARNT2, aryl hydrocarbon receptor nuclear translocator 2, elicited an induced expression at 24 and 48h in contrast to *ARNT* whose expression remained unchanged (data not shown). Although the ability of ARNT2 to participate as a heterodimer with AhR in the *CYP1A1* gene expression is controversial, it should play a role in the response to hypoxia and anti-oxidant activities (Hankinson, 2008).

In addition to *CYP1A1* and *CYP1B1* encoding the type I enzymes of detoxification, the induction of genes encoding phase II enzymes was also identified at different kinetic time points (Table 4). Among them, *UGT1A8* and *UGT1A6* encode glucuronosyltransferases, and *SULT2B1* encodes a sulfotransferase. It has been shown that phase II enzymes were highly expressed and inducible by 3-methylcholanthrene, an homologous to  $\beta$ NF, in the human keratinocyte NCTC 2544 cell line (Gelardi et al., 2001).

It is known (Gelardi et al., 2001) that such a xenobiotic response is accompanied by the initiation of an inflammatory state that indeed is detected in our present study (Fig. 2). This inflammation might result from the expression of *IL1B* and *IL1A* that was found strongly induced from 6h onwards (Table 4). In addition, an induced expression of several genes encoding IL1B-related receptors such as *IL1RN* and *IL1R2* was detected at all time points. Interestingly, *IL1RN* was shown to inhibit the interaction between *IL1R2* and *IL36G* whose expression was seen consistently highly induced (Table 4). Binding of IL1B to *IL1R2* was reported to inhibit the expression of *CTGF* (Zhu, 2010) that indeed was found to be repressed by  $\beta$ NF at 6, 24 and 48h. Furthermore, IL1B, through the involvement of *IRAK2*, interleukin 1 receptor associated kinase 2, can

trigger the formation of an active NFκB complex (Fig. 2) that can be formed following the expression of *RELB* encoding the p65 subunit at 6 and 24h. The p65 subunit interacts with p50 to result in a heterodimer enabling to bind the NFκB motif present in the promoter of IL1α and TNF-α target genes. As shown in Figure 2, several genes encoding others NFκB related factors (NFKBIZ, NFKBIA, NFKBIE) and NFκB target genes including inflammatory genes such as *TNF* and other interleukins and chemokines (Fig. 2) were found induced from 6h of treatment (Table 4). NFKBIZ, NFKBIA, NFKBIE are members of the nuclear I kappa B protein family and are regulators of the NFκB activity (Ghosh and Karin, 2002). While NFKBIZ stimulates the production of IL6 (Trinh et al., 2008), NFKBIE inhibits NFκB by trapping it in the cytoplasm, and NFKBIA interacts with REL dimers to inhibit NFκB /REL complexes, which are involved in inflammatory response (Scherer et al., 1995).

In addition, several genes such as *MMP3*, *MMP9* and *MMP10* encoding molecules involved in the extra cellular matrix (ECM) degradation and remodeling (Fig. 2), known to be induced by IL1α, were upregulated by βNF (Table 4).

Moreover, an induced expression of *CLDN1* (Claudine 1), *CLDN4* (Claudine 4) and *OCN* (Occludine) genes involved in the formation of tight junctions was observed from 6h onwards (Table 4). Such an induction might be part of a protective effect in response to βNF treatment to preserve the integrity of the cell-cell interactions.

On the other hand, reactive oxygen species (ROS) are produced through the metabolism of AhR by CYP activities (Hrycay and Bandiera, 2015). Excessive oxidative damage



resulting from ROS production is controlled through the activation of a protective process. Such a process is illustrated by the induction of a significant number of genes controlled at a transcriptional level by the antioxidant response element (ARE), a target of NRF2. However all these genes were induced only at 24 and 48h. Free of its inhibitor KEAP1, NRF2, can be activated by AP1 (Fig. 2), a key transcription factor for the survival and differentiation of keratinocytes (Mehic et al., 2005). Then, the resulting complex translocates into the nucleus and binds to the ARE motif present in genes (Table 4) whose encoded products such as MAFF, MAFFB and MAFG are reported to interact with NRF2. This signaling pathway will induce the gene expression of cytoprotective/antioxidant markers (SOD2, NQO1, AKR1C1, AKR1C3, HMOX1, MT1F and MT1X). Noteworthy, the induction of all these genes in response to NRF2 activation can be achieved only after 24h of treatment (Table 4). Moreover while NRF2 is stabilized by SQSTM1/p62 (sequestosome 1), which is itself a transcriptional target of NRF2 (Greninger, 1986), it was reported that the NRF2 signaling pathway can be downregulated by NFE2L3/NRF3 (Sankaranarayanan and Jaiswal, 2004). Another negative control of this signaling pathway can depend from IER3, immediate early response 3, whose overexpression was shown to attenuate NRF2 activation (Stachel et al., 2014). Thus, our results suggested that at the transcriptional level, the NRF2-dependent antioxidant response initiated in response to  $\beta$ NF was occurring after inflammation and was potentially controlled by a negative regulatory loop (Fig. 2).

That  $\beta$ NF initiated early the proliferation of keratinocytes is supported by the expression of *IGFBP3* at 6h that was maximal at 24h (Table 4). Indeed, it has been shown that IGFBP3 was highly expressed in proliferative keratinocytes and its overexpression inhibited their proliferation modulating the early stages of their differentiation (Edmondson et al., 2005).

Finally, the ultimate process triggered by  $\beta$ NF seemed to regenerate the outermost layer of skin (Fig. 3) as illustrated by the expression of KRTDAP, involved in keratinocyte differentiation and the maintenance of stratified epithelia, as well as of different types of keratin genes (*KRT1*, *KRT6B*, *KRT13*, *KRT19*, *KRT19P2*, *KRT31*, *KRT34*, and *KRT78*) in response to the treatment at 24 and 48h. This effect of  $\beta$ NF was also supported that the induced expression of genes encoding components of the cornified envelope such as CNFN and cornifelin, (Table 4) as well as proteins that belong to the families of the small prolin rich proteins and the S100 calcium binding proteins. As for *IVL* (involucrin), all the corresponding genes are clustered within a locus located at chromosome 1q21 called the epidermis differentiation complex (Fig. 3). Most of these genes were induced from 6 h onwards (Table 4) and plays a crucial role in differentiation and maturation of the human epidermis (Henry et al., 2012; Kypriotou et al., 2012). However, even carried on the same locus, expression of the genes encoding the S100 calcium binding proteins was delayed in comparison to those encoding the small prolin rich proteins and involucrin (Table 4).

## **Conclusion**

Our results confirmed that keratinocytes are able to respond to  $\beta$ NF that at an early stage triggers an efficient transcriptional program resulting in a xenobiotic response, an inflammation and the proliferation of keratinocytes. Further on, these processes are followed by the induction of transcriptional activities leading to an anti-oxidative stress response and to the synthesis of components of the cornified envelope in the course of keratinocyte terminal differentiation. To the best of our knowledge, we have provided the first evidence of the different processes induced overtime sustaining the protective effect initiated in primary keratinocyte cells upon  $\beta$ NF exposure.

### **Conflict of interest**

The authors declare no financial conflict of interest that might be construed to influence the results or interpretation of the manuscript.

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## **Legends of figures**

**Figure 1.** Induction of EROD activity in HEK exposed to a concentration range (0.01 - 10  $\mu$ M) of  $\beta$ NF for 48 or 72 hours. The results, generated in two independent experiments, are expressed as the mean of ratios  $\pm$  S.D. between treated versus untreated conditions (n = 12). P values were obtained by comparing each conditions activity with untreated control activity in a Student's t-test. \* p <0.05, \*\* p <0.01, \*\*\* p <0.001.

**Figure 2.** Schematic representation of the xenobiotic/anti-oxidant response, inflammation, triggered by  $\beta$ NF deduced from PredictSearch<sup>®</sup> analysis in HEK cells. Legends are indicated at the bottom. The xenobiotic response is surrounded by dots and the anti-oxidative response by broken lines.

**Figure 3.** Schematic representation of the effects triggered by  $\beta$ NF on skin barrier. Legends are indicated at the bottom.

## Tables

**Table 1:** Effects of  $\beta$ -naphthoflavone treatments on the viability of HEK cell evaluated by the MTT method. The values represent the means of three independent experiments  $\pm$  S.D. and correspond to the variation expressed as a percentage between treated and untreated conditions. \* P <0.05; \*\* P <0.01; \*\*\* P <0.001, compared with the control by an ANOVA test followed by a Dunnett post-test (n = 12).

Inducer Concentration	Time of exposure		
	14h	24h	48h
0,01 $\mu\text{M}$	108,9 $\pm$ 4,95	101,5 $\pm$ 9,20	104,1 $\pm$ 11,73
0,05 $\mu\text{M}$	106,6 $\pm$ 13,80	102,7 $\pm$ 6,28	102,1 $\pm$ 5,79
0,1 $\mu\text{M}$	96,19 $\pm$ 10,23	88,41 $\pm$ 5,17	100,3 $\pm$ 7,32
0,5 $\mu\text{M}$	99,91 $\pm$ 7,17	93,54 $\pm$ 9,21	99,5 $\pm$ 8,46
1 $\mu\text{M}$	97,11 $\pm$ 9,38	88,54 $\pm$ 5,78	93,83 $\pm$ 5,07
5 $\mu\text{M}$	94,84 $\pm$ 4,69	77,64 $\pm$ 5,50 ***	89,21 $\pm$ 5,43
10 $\mu\text{M}$	82,43 $\pm$ 9,22 *	73,33 $\pm$ 9,01 ***	83,34 $\pm$ 8,15 *
100 $\mu\text{M}$	70,46 $\pm$ 9,15 ***	61,15 $\pm$ 10,73 ***	34,61 $\pm$ 12,29 ***

**Table 2.** Comparison of CT modulation and fold induction ratios of selected CYP genes. RT-qPCR was performed on total RNAs extracted from HEK cells exposed to  $\beta\text{NF}$  for 48 hours. The results are expressed as means of CT  $\pm$  S.D. obtained in three independent experiments. ND indicates that the amplification was not detected.

		GAPDH	CYP1A1	CYP1A2	CYP3A	CYP2B6
$\beta\text{NF}$	-	18.5 $\pm$ 0.5	29.8 $\pm$ 1.1	30.7 $\pm$ 0.5	33.7 $\pm$ 0.8	35.3 $\pm$ 0.4
	+	17.9 $\pm$ 0.5	22.9 $\pm$ 0.1	24.6 $\pm$ 0.6	33.7 $\pm$ 1.1	35.7 $\pm$ 0.8
<b>FC.</b>		1.0	75	42	ND	ND

**Table 3.** Distribution of genes modulated in HEK cells exposed to  $\beta\text{NF}$  at distinct consecutive times according to the transcriptomic analysis.

Times of treatment	○ Number of induced genes (FC. $\geq 1.5$ )	Number of repressed genes (FC. $\leq 0.6$ )
6h-24h	128	7
24h-48h	446	96
6h-24h-48h	84	3

**Table 4.** List of selected genes, whose expression is modulated by  $\beta$ -naphthoflavone.

	Gene description	Fold-induction		
		6h	24h	48h
<i>CYP1A1</i>	Cytochrome P450, family 1, subfamily A, polypeptide 1	9.5	98.3	155
<i>CYP1A2</i>	Cytochrome P450, family 1, subfamily A, polypeptide 2	2.0	8.1	19.6
<i>CYP1B1</i>	Cytochrome P450, family 1, subfamily B, polypeptide 1	13.2	43.3	44.9
<i>CYP27A1</i>	Cytochrome P450, family 27, subfamily A, member 1	1.0	1.7	1.7
<i>CYP27B1</i>	Cytochrome P450, family 27, subfamily B, member 1	1.3	1.9	1.7
<i>CYB5A1</i>	Cytochrome b5 type A	1.0	1.5	2.7
<i>CYB5R2</i>	Cytochrome b5 reductase 2	1.0	1.6	2.6
<i>AHR</i>	Aryl hydrocarbon receptor	0.8	0.8	0.4
<i>ARNT2</i>	Aryl hydrocarbon receptor nuclear translocator	1.0	1.5	1.6
<i>UGT1A6</i>	UDP glucuronosyltransferase 1 family, polypeptide A6	1.2	1.6	2.1
<i>UGT1A8</i>	UDP glucuronosyltransferase 1 family, polypeptide A8	1.2	1.9	2.0
<i>SULT2B1</i>	Sulfotransferase family, cytosolic, 2B, member 1	1.6	5.7	3.4
<i>IL1B</i>	Interleukin 1, beta	2.9	7.6	4.2
<i>IL1A</i>	Interleukin 1, alpha	2.1	2.4	1.0
<i>IL1RN</i>	Interleukin 1 receptor agonist	13.2	43.3	44.9
<i>IL1R2</i>	Interleukin 1 receptor type 2	2.0	13.1	13.1
<i>IL36G</i>	Interleukin 36, gamma	14.0	51.3	18.2
<i>CTGF</i>	Connective tissue growth factor	0.6	0.5	0.1
<i>RELB</i>	V-rel avian reticuloendotheliosis viral oncogene homolog B	1.6	2.1	0.9
<i>IRAK2</i>	Interleukin 1 receptor associated kinase 2	2.2	4.3	2.8
<i>NFKBIZ</i>	NFKB inhibitor zeta	1.7	2.3	1.3
<i>NFKBIA</i>	NFKB inhibitor alpha	2.1	2.9	2.1
<i>NFKBIE</i>	NFKB inhibitor epsilon	1.4	2.1	2.0
<i>TNF</i>	Tumor necrosis factor	4.9	5.3	5.2
<i>IL6</i>	Interleukin 6	6.4	13.2	1.0
<i>IL11</i>	Interleukin 11	1.6	1.7	0.9
<i>LTB</i>	Lymphotoxin beta	2.1	2.3	1.4
<i>CXCL2</i>	Chemokine (C-X-C motif) ligand 2	2.9	3.7	1.2
<i>CXCL3</i>	Chemokine (C-X-C motif) ligand 3	4.6	3.4	1.0
<i>IL20</i>	Interleukin 20	3.2	6.4	1.9
<i>IL24</i>	Interleukin 24	5.3	16.2	5.0
<i>IL23A</i>	Interleukin 23, alpha subunit p19	1.6	4.3	2.8
<i>IL32</i>	Interleukin 32	1.8	9.9	5.7
<i>CXCL11</i>	C-X-C motif chemokine ligand 11	1.5	7.8	2.4
<i>MMP3</i>	Matrix metalloproteinase 3	2.8	2.2	0.7
<i>MMP9</i>	Matrix metalloproteinase 9	1.9	9.7	3.0
<i>MMP10</i>	Matrix metalloproteinase 10	1.6	8.2	0.7
<i>PLAT</i>	Plasminogen activator, tissue type	1.5	4.0	5.5
<i>PLAU</i>	Plasminogen activator, urokinase	1.4	1.8	2.2
<i>PLAUR</i>	Plasminogen activator, urokinase receptor	2.5	3.6	3.1
<i>TIMP2</i>	TIMP metalloproteinase inhibitor 2	1.1	3.1	4.6
<i>OCLN</i>	Occludin	2.2	11.1	1.1
<i>CLDN1</i>	Claudin 1	2.3	3.1	1.0
<i>CLDN4</i>	Claudin 4	2.4	5.6	6.8
<i>MAFF</i>	V-maf avian musculoaponeurotic fibrosarcoma oncogene homolog F	2.0	1.8	2.0
<i>MAFB</i>	V-maf avian musculoaponeurotic fibrosarcoma oncogene homolog B	1.8	1.8	1.2
<i>MAFG</i>	V-maf avian musculoaponeurotic fibrosarcoma oncogene homolog G	1.7	1.5	1.5
<i>IER3</i>	Immediate early response 3	1.9	2.0	2.0
<i>NFE2L3</i>	Nuclear factor, erythroid 2 like 3	1.0	1.6	2.4
<i>SQSTM1</i>	Sequestosome 1	1.4	2.1	1.9
<i>ALDH3A1</i>	Aldehyde dehydrogenase 3 family A1	1.1	1.7	6.8
<i>HMOX1</i>	Heme oxygenase 1	1.3	1.9	2.6
<i>AKR1C1</i>	Aldo-keto reductase family member C1	0.9	3.6	5.9
<i>AKR1C3</i>	Aldo-keto reductase family member C3	0.9	3.0	5.2
<i>NQO1</i>	NAD(P)H dehydrogenase, quinone 1	1.0	1.5	1.8
<i>SOD2</i>	Superoxide dismutase 2, mitochondrial	1.3	5.7	4.4
<i>MT1F</i>	Metallothionein 1F	1.1	1.5	1.5
<i>MT1X</i>	Metallothionein 1X	1.0	1.5	4.4
<i>KRT1</i>	Keratin 1, type II	0.5	1.8	6.7
<i>KRT6B</i>	Keratin 6B, type II	1.1	2.7	2.4
<i>KRT13</i>	Keratin 13	1.1	1.5	2.5
<i>KRT19</i>	Keratin 19, type I	0.9	1.5	2.3
<i>KRT31</i>	Keratin 31, type I	1.1	2.1	2.0
<i>KRT34</i>	Keratin 34, type I	2.4	6.1	2.7
<i>KRT78</i>	Keratin 78, type II	1.0	1.4	3.3
<i>KRTDAP</i>	Keratinocyte differentiation associated protein	0.8	3.6	6.7
<i>IGFBP3</i>	Insulin like growth factor binding protein 3	1.9	10.5	1.5
<i>CNFN</i>	Cornefelin	1.1	2.7	11.1
<i>IVL</i>	involucrin	1.6	4.0	9.5
<i>SPRR1A</i>	Small proline-rich protein 2A	1.0	5.1	7.6
<i>SPRR1B</i>	Small proline-rich protein 2A	1.0	2.7	3.3
<i>SPRR2A</i>	Small proline-rich protein 2A	1.8	9.1	3.6
<i>SPRR2D</i>	Small proline-rich protein 2D	2.0	8.2	3.0
<i>SPRR2E</i>	Small proline-rich protein 2E	2.0	8.7	3.5
<i>SPRR2F</i>	Small proline-rich protein 2F	4.6	3.4	1.0
<i>S100A7</i>	S100 calcium binding protein A7	1.2	3.0	6.0
<i>S100A4</i>	S100 calcium binding protein A4	1.0	1.7	3.6
<i>S100A8</i>	S100 calcium binding protein A4	1.1	11.5	5.3
<i>S100A9</i>	S100 calcium binding protein A4	1.1	16.2	10.4