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EXPRESSION OF NUCLEAR RECEPTORS (AhR, PXR, CAR) AND TRANSCRIPTION FACTOR (Nrf2) IN HUMAN PAROTID GLAND

AGNIESZKA DROŹDZIK[1,*], ROBERT KOWALCZYK[2], ELŻBIETA URASIŃSKA[3] and MATEUSZ KURZAWSKI[4]

[1]Department of Periodontology, Pomeranian Medical University, Powstańcow Wlkp. 72 St., 70-111 Szczecin, Poland
[2]Department of Maxillofacial Surgery, Pomeranian Medical University, Szczecin, Poland
[3]Department of Pathomorphology, Pomeranian Medical University, Szczecin, Poland
[4]Department of Pharmacology, Pomeranian Medical University, Szczecin, Poland

Abstract: Nuclear receptors and transcription factors coordinate expression of many genes, and regulation of their expression determines cellular response to various endo- and exogenous factors. There is paucity of data regarding expression of nuclear receptors and factors in salivary glands. In the present study, a focus was placed on human parotid gland expression of aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR, *NR1I2*), constitutive androstane receptor (CAR, *NR1I3*) and nuclear factor E2–related factor 2 (Nrf2). Parotid salivary tissue was obtained from patients undergoing the gland dissection. Quantitative real-time PCR and immunohistochemical staining were used for expression studies. The highest mRNA expression was documented for *NFE2L2* coding for Nrf2. Lower expression was seen in the case of *AHR* gene coding for AhR. *PXR* was constitutively present at very low level and *CAR* expression was below the limit of quantification. Immunohistochemical evaluation of the parotid gland specimens revealed cytoplasmic Nrf2 expression in striated duct cells as well as within myoepithelial cells. Acinar cells were mostly negative for Nrf2. Expression of AhR was found within the cytoplasm in striated duct cells. Acinar and myoepithelial cells were negative for AhR. Having in mind their role in regulating function of many enzymes and transmembrane transporters, expression of these factors seem play a role in salivary gland physiology, pathology as well as drug transport and metabolism.

Keywords: AhR, PXR, CAR, Nrf2, salivary gland

Nuclear receptors and transcription factors coordinate expression of many genes, and regulation of their expression determines cellular response to various endo- and exogenous factors. Therefore, description of their expression in a tissue gives an insight into its potential regulatory pathways, and ability to respond to endo- and exogenous stimuli. There is paucity of data regarding expression of nuclear receptors and factors in human salivary glands. In the present study a focus was placed on human parotid gland expression of aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR, *NR1I2*), constitutive androstane receptor (CAR, *NR1I3*) and nuclear factor E2–related factor 2 (Nrf2, *NFE2L2*).

Nrf2 regulates expression of genes containing antioxidant-response elements in a promoter region, and plays a key role among all redox-sensitive transcription factors protecting cells from oxidative insult. Along with Keap1, Nrf2 forms one of the major cellular antioxidant systems (Nrf2-Keap1), which acts as a molecular sensor of disturbances in cellular homeostasis, and is activated by endo- and exogenous factors (1). It was found that knockout (nrf2-/-) mice were characterized by an enhanced susceptibility to the toxicities associated with various xenobiotics and environmental stresses. These animals exhibit no significant early developmental phenotype, but aged mice develop vacuolar leukoencephalopathy and lupus-like autoimmune symptoms. Furthermore, Nrf2-null animals display low basal and/or inducible expression of cytoprotective genes (e.g., glutathione S-transferase, thioredoxin reductase, heme oxygenase, metallothionein 1, superoxide dismutase, NAD(P)H quinone reductase 1) in a variety of tissues, including liver, lung, gastrointestinal tract, skin and bladder (reviewed in

* Corresponding author: e-mail: agdro@pum.edu.pl; phone: +48-914661767; fax: +48-914661744

ref. 2). Animal studies and human data support observations from knockout mice (nrf2-/-), showing that Nfr2 is an important player in pathologies related to oxidative stress, immunological dysfunction, dysregulated inflammatory response. i.e., tumorigenesis (3), inflammatory and autoimmune diseases (4), drug induced side effects (3), cardiovascular pathology (5), metabolic and endocrine diseases (6) and many other pathologies.

Nuclear receptors AhR, CAR and PXR were initially characterized as xenosensors, but now it is evident that they can also trigger pleiotropic effects on many tissues function. These nuclear receptors regulate activity of genes involved in xenobiotic and drug metabolism and transport. The existence of crosstalk between these xenosensors and other nuclear receptors or transcription factors (including Nrf2) controlling endogenous signaling pathways was revealed (7).

AhR controls transcription of genes involved in phase I and phase II metabolic enzymes, such as cytochrome P450 1A1, cytochrome P450 1B1, NAD(P)H quinone oxidoreductase 1 and aldehyde dehydrogenase 3 (ALHD3A1). There are two major categories of environmental compounds that activate AhR signaling: halogenated aromatic hydrocarbons (e.g., TCDD) and polycyclic aromatic hydrocarbons (e.g., benzo(a)pyrene) (8). Induction of xenobiotic metabolizing enzymes following AhR activation is considered, at least in part, as an adaptive response of the organism to its environment, which could decrease the potential toxicity of foreign chemicals. Recent discoveries pointed out novel AhR functions, i.e., its involvement into multiple aspects of physiology, such as reproduction, innate immunity and tumor suppression (9).

CAR and PXR are other two very important nuclear receptors, which regulate gene transcription of enzymes and membrane transporters in response to exo- and endogenous compounds, forming a line cellular defense against e.g., drugs and other toxins. CAR and PXR regulate a largely overlapping set of xenobiotic metabolizing genes: CYPs (i.e., CYP3A4, CYP2B6, CYP2Cs, CYP2A6), UGTs (i.e., UGT1A1, UGT1A6, and UGT1A9), glutathione transferases and sulfotransferases as well as drug transporters such as MDR1, and OATPs (reviewed in 10). On the other hand, CAR displays unique activation for CYP1A, AHR and FMOC5, whereas PXR for MRP3, CYP4F12, CYP7A and CAR (11). Together, CAR and PXR orchestrate an adaptive network in combating toxic byproducts derived from both endogenous and exogenous chemicals.

The nuclear factors and receptors, such as Nrf2, AhR, CAR and PXR, being sensors responding to endo- and exogenous compounds, due to their involvement in regulation of genes play an important role in drug metabolism and transport, detoxifying processes and many physiological processes. Therefore, it was decided in the present study to evaluate their expression in human parotid gland as they can participate in homeostasis of the gland, and drug transport and metabolism as well as pathological processes, e.g., carcinogenesis.

Table 1. Patients characteristics.

Sex	Age	Diagnosis	Medication at the time of sampling	Other diseases
F	62	Mixed tumor	estazolam, ketamine, cephotaxim, tramadol	-
F	61	Mixed tumor	estazolam, ketamine, amoxicilline, pethidine	-
F	30	Mixed tumor	midazolam, ketamine, pethidine, cephotaxim	-
M	53	Mixed tumor	midazolam, ketamine, pethidine, cephotaxim, enalapril	Hypertension
M	55	Mixed tumor	estazolam, ketamine, morphine, cephazoline	-
F	58	Mixed tumor	estazolam, ketamine, cefuroxime, tramadol	-
F	40	Mixed tumor	estazolam, ketamine, cefuroxime, tramadol	-
F	47	Mixed tumor	estazolam, ketamine, cephazoline, tramadol	-

MATERIALS AND METHODS

Parotid gland specimens

Parotid salivary glands were from healthy tissues of parotid salivary glands obtained from 8 patients undergoing the gland dissection. Control liver tissue samples were obtained from nontumoral liver tissue from 6 patients with metastatic liver tumors that required surgical resection. A part of each specimen was immediately preserved in RNAlater (Applied Biosystems, USA) for RNA analysis and adjacent tissue was embedded in formalin for immunohistochemistry. Detailed patient characteristics is provided in Table 1. The study protocol was approved by local ethics committee, and patients gave informed consent.

RNA expression study

Total RNA was extracted from 50-100 mg tissue samples (RiboPure Kit, Ambion, USA). Quantity and quality of isolated RNA was evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA), concentrations ranged from 500 to 900 ng per µL, and the ratio of the absorbance at 260 and 280 nm ($A_{260/280}$) was over 1.9 in all samples. Subsequently, cDNA was prepared from 1 µg of total cellular RNA [in 20 µL of reaction volume - RETROscript First Strand Synthesis Kit (Ambion, USA)] with oligo-dT primers, according to the manufacturer's instructions. Pre-validated TaqMan Gene Expression Assay (assay IDs: *AHR* – Hs00169233_m1, *NFE2L2* Hs00232352_m1, *NR1I2* (*PXR*) Hs00243666_m1 and *NR1I3* (*CAR*) Hs00901571_m1, TaqMan GE Master Mix (Applied Biosystems, USA) and 1.5 µL of cDNA for each reaction mix of 15 µl were used for quantitative real-time PCR [7500 Fast Real-Time PCR System (Applied Biosystems, USA)]. All the assays were guaranteed for their PCR efficiency of 100 ± 2%. Each sample was analyzed simultaneously in three technical replicates, and mean Ct values were used for further analysis.

Calculations were performed using the ΔΔCt relative quantification method, using 7500 Fast Real-Time PCR System Software (Applied Biosystems, USA) (12). The thresholds were set manually to compare data between runs, and Ct values were extracted. All Ct values for each sample were normalized to the mean value obtained for two house-keeping genes, processed in the same run: *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and *ACTB* (β-actin), as those genes are commonly used as endogenous controls in gene expression studies. Fold change between groups was calculated from the means of the logarithmic expression values.

Immunohistochemical staining

Formalin-fixed, paraffin-embedded 5 µm sections from parotid glands were deparaffinized, rehydrated and immersed in pH 9.0 buffer. Heat-induced antigen retrieval was performed in a pressure cooker (Pascal, Dako, Denmark) at 120°C for 3 min. Slides were incubated with primary rabbit polyclonal anti-Nrf2 antibody (ab31163, Abcam, USA, dilution 1:50) or rabbit polyclonal anti-AhR antibody (ab49351, Abcam, USA, dilution 1:50) for 30 min at room temperature and immunostained with a Dako Envision+ kit for 30 min, AEC+ as a chromogen and hematoxylin as counterstain. Normal mouse immunoglobulins were substituted for primary antibodies as negative controls.

RESULTS

Expression of nuclear factor and nuclear receptors was observed in the human parotid salivary gland, both at mRNA and protein level (evaluated by immunohistochemistry). From all nuclear receptors and nuclear factor studied the highest

Table 2. Relative expression of the studied genes in the human parotid gland, compared with liver tissue samples.

Gene	Relative expression	SD	p
AHR	0.04128	0.01655	0.0006
NFE2L2	0.41487	0.10755	0.0079
NR1I2 (PXR)	0.00063	0.00029	0.0007
NR1I3 (CAR)	BLQ	-	-

Numbers represent relative mRNA expression in salivary glands (n = 8) given as a fraction of values obtained for human liver samples (n = 6); BLQ – below the limit of quantification; SD - standard deviation; p values obtained by means of Mann-Whitney U-test (salivary gland *vs*. liver).

expression was documented for *NFE2L2* gene coding for Nrf2 (mean C_T value = 25.3). Lower expression was seen in the case of *AHR* gene coding for AhR (mean C_T value = 28.6). The other two nuclear receptors transcripts, i.e., *NR1I2 (PXR)* and *NR1I3 (CAR)* were present at very low level (*NR1I2*, mean C_T value = 34.5) or below the limit of quantification method (*NR1I3*). Results of gene expression in salivary gland, presented as relative expression compared with liver tissue samples are presented in Table 2.

Therefore, in the next step of the study, i.e., in the immunohistochemical localization of the receptors only Nfr2 (Fig. 1) and AhR (Fig. 2) were evaluated. Immunohistochemical evaluation of the parotid gland specimens revealed cytoplasmic Nrf2 expression in striated duct cells as well as within myoepithelial cells. Acinar cells were mostly negative for Nrf2. Expression of AhR was found within the cytoplasm in striated duct cells. Acinar and myoepithelial cells were negative for AhR.

DISCUSSION

In spite of nuclear receptors and factors involvement in regulation of many physiological processes as well as pathology there is no available data on their expression and distribution in human salivary glands. The data gathered during the present study revealed that within the human parotid gland tissue an expression of some nuclear receptors and factors can be found, i.e., relatively high expression of *NFE2L2*, weaker of *AHR*, very low of *PXR* and lack of *CAR*. These results are in keeping with the Nishimura et al. study (13), who reported the same range of *PXR* expression (very low), lack of *CAR* expression at mRNA level in salivary gland (without definition of the gland evaluated).

Our report is the first one showing expression of Nrf2 and AhR in human parotid glands. The immunohistochemical localization of the latter two indicates that both nuclear factor Nrf2 and nuclear receptor AhR proteins are expressed in striated duct cells, whereas myoepithelial cells were positive for Nrf2 only. Acinar cells were not stained by antibodies against Nrf2 and AhR. Both Nfr2 and AhR control an adaptive network of enzymes regulating oxidative stress, inflammatory response, immune function and drugs' and toxins' metabolism and transmembrane transport. Their expression in salivary ducts suggests that Nrf2 and AhR may constitute a functional barrier, which would protect oral tissues from toxic products in salivary duct cells. The protective role of Nrf2 in other barriers was documented for blood-brain barrier (14), intestines (15), lungs (16) and skin (17). High Nrf2 expression is also seen in the liver (reviewed in 18). Likewise, AhR is expressed in the liver, where it coordinates gene expression active in detoxification and drug metabolism (reviewed in 18). Similar functions AhR plays in other tissues, where constitutive expression of the receptor was defined, i.e., lung, kidney, spleen and placenta (reviewed in 19). In those tissues AhR induced by its ligands, i.e., polycyclic aromatic

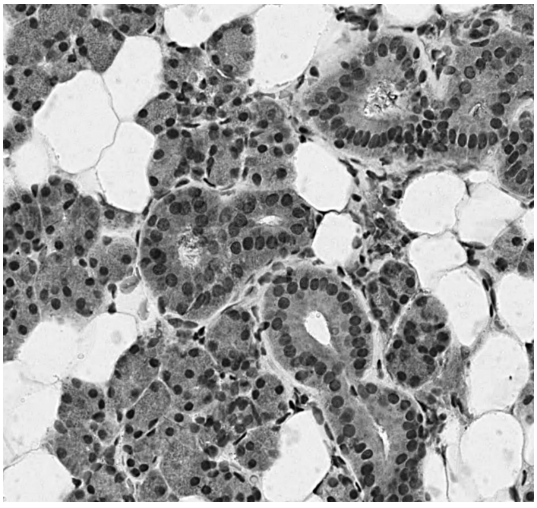

Figure 1. Immunohistochemical expression of Nrf2 in the parotid gland. Striated duct cells as well as some myoepithelial cells reveal strong positive cytoplasmic staining. Acinar cells are mostly negative

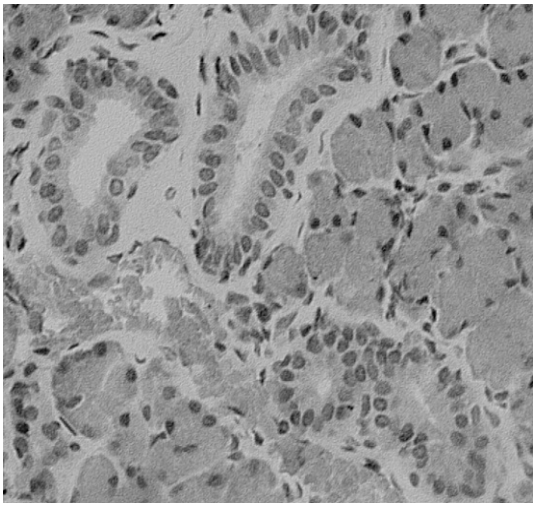

Figure 2. Immunohistochemical expression of AhR in the parotid gland. Note the weak positive cytoplasmic expression in striated duct cells. Acinar and myoepithelial cells are negative

hydrocarbons, halogenated aromatic hydrocarbons, indole derivatives, promotes activation of genes implicated their metabolism, and thus limiting their detrimental cellular effects (e.g., procarcinogenic effects). The functional role of both systems in salivary glands can be supported by expression within the glands, of some AhR and Nrf2 dependent enzymes, namely for AhR – NAD(P)H quinone oxidoreductase 1, aldehyde dehydrogenase 3, CYP1A1 and CYP1B1 (20,21) as well as for Nrf2 – NAD(P)H quinone oxidoreductase 1 (20). So, activity of both Nrf2 and AhR may be very important in preserving salivary gland homeostasis, and play important role in pathology (e.g., carcinogenesis, autoimmune gland destruction, gland dysfunction in metabolic diseases, drug transport and toxicity etc.). In our study, similarly to report of Nishimura et al. (13), a very weak constitutive expression of *PXR* was observed. In spite of low PXR constitutive expression, its role in the parotid gland physiology cannot be neglected as our previous study have documented expression in salivary gland of a transporter P-glycoprotein (22) encoded by *MDR1* gene, which expression is regulated by PXR. Also expression of other genes controlled by PXR was reported. i.e., *MDR1*, *MRP1* (at mRNA level) within salivary gland by Nishimura et al. (23), and ductal expression of MRP1 by Uematsu et al. (24). Therefore, the presence of transporters regulated by PXR in human parotid gland suggests that this nuclear receptor may play an important role in regulation of membrane trafficking.

In conclusion, it can be stated that it is the first paper documenting human parotid gland constitutive expression of nuclear factor Nrf2 and nuclear receptor AhR defining their cellular localization predominantly within striated duct cells. We have also confirmed low level expression of *PXR* nuclear receptor. Having in mind their role in regulating function of many enzymes and transmembrane transporters, expression of these factors seems to be important in salivary gland physiology and pathology.

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