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N–nitrosodiethylamine cytochrome P450 induction and cytotoxicity evaluation in primary cultures of rat hepatocytes

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ABSTRACT

The primary routes of potential human exposure to *N*-nitrosodiethylamine (NDEA) are ingestion, inhalation, and dermal contact. Air, diet and smoking contribute to potential human exposure at levels of a few µg of NDEA/day. Potential exposure depends on the ability of the nitrosamines to migrate from the product into the body. The first step in the metabolic degradation of NDEA by cytochrome oxidase (CYPs) enzymes is the introduction of a hydroxyl group and in human esophage and liver CYP2A3 and CYP2E1 participate on this metabolism. Measuring cytotoxicity in female rat primary hepatocytes cultures, were used to understand the CYP induction and metabolization correlated with low NDEA concentrations. We observed that NDEA at different concentrations in the absence of CYPs inducers, was able to induce CYP2B1, CYP2B2, CYP2E1, CYP3A1 and CYP4A3. A positive NDEA synergistic effect on the levels of mRNA, was observed in the presence of pyrazole (300 µM) for CYP2B1 and CYP2B2 and for pregnenolone 16-α carbonitrile (0.15 µM) for CYP2E1. Negative NDEA synergistic effects were observed for ethanol (0.3%) for CYP3A1, pyrazol (300 µM) for CYP2A1 and CYP2E1, and phenobarbital (1 mM) for CYP2A1. These facts are extremally important once that these metabolites can be directly related to the primary DNA lesions. We consider that studies to elucidate the biological factors that determine the shape of the dose-response curve are crucial for low-dose extrapolations of risk.

Keywords: *N*-Nitrosodiethylamine; Cytochrome P450; Cytotoxicity; Primary Culture; Hepatocyte; Real-Time PCR.

1. INTRODUCTION

Cytochrome oxidase (CYPs) form a superfamily of haemthiolate proteins present in prokaryotes and throughout the eukaryotes. CYPs act as mono-oxygenases, with functions ranging from the synthesis and degradation of endogenous steroid hormones, vitamins and fatty acid derivatives ("endobiotics") to the metabolism of foreign compounds such as drugs, environmental pollutants, and carcinogens ("xenobiotics") [1]. The majority of the CYP isoforms are found in the liver, however other extra-hepatic sites include the center nervous system, gastrointestinal tract, kidney, lungs, and adrenal glands [2].

CYP gene expression is regulated by several factors, as gender, microsomal enzyme inducers, age, diet, and hormones. Differences in the amounts and intrinsic capacities of CYP forms to metabolize a particular drug or chemical may influence profoundly drug-drug interactions, drug or carcinogen activation and detoxification contributing to the development of cancer, Parkinson's disease, and adrenal hyperplasia [3].

N-nitrosodiethylamine (NDEA) is activated by cytochrome P450 enzymes, resulting in ethylation of N and O atoms of most bases from DNA. The N7, and O6 positions of guanine are preferable ethylated, and a lower level of ethylation is also observed at the O4 position of thymine [4]. O^6-ethylguanine and O^4-ethylthymine, if not repaired, will lead to mutation and tumour formation in superior animals [5]. NDEA is able to produce tumours in many species of animals and in a variety of organs, requiring metabolic activation through P450-catalyzed α-hydroxylation, generating unstable metabolites that will alkylate the DNA at the site of activation, in order to induce tumors [6]. Till the present, there is no evidence that NDEA is a CYP inducer or if CYP's in-

duction can increase or decrease others previously induced CYPs.

The presence of NDEA in foodstuffs has been a subject of several reviews [7-11]. Druckrey and Preussmann (1962) postulated that NDEA formation could arise in tobacco smoke via the interaction of nitrogen oxides and tobacco amines [12]. Additionaly, mechanisms for the *in vivo* formation of NDEA may involve chemical and enzymatic nitrosation especially dependent on the presence of both nitrate and nitroreductases [13-16].

In Wistar rats, a commonly used experimental model for oesophageal carcinogenesis, NDEA induces tumours at low doses. When higher doses are given for a shorter period, tumours originate mainly in the liver [5]. Reasons for such interorgan differences are currently unknown but it has been hypothesized that this could be due to different enzymes being responsible for the metabolic activation of NDEA [17,18].

The differences in the amounts and intrinsic capacities of CYP forms to metabolize a particular chemical may influence: drug-drug interactions, drug or carcinogen activation and detoxification. This knowledge has contributed to an increase in the scientific literature on CYP-dependent drug metabolism. Predicting the ability of a drug, like NDEA, to modulate CYP expression at an early stage may permit the identification of alternative noninducing chemical structures. For the pharmaceutical industry the potential induction of various CYPs by drugs candidates, is important once they can lead to toxicity or reduce drug-drug interactions efficacy.

During the last few years, there has been a great interest in developing rapid and simple tests to identify the effects of exposure to environmental agents that can induce DNA damage. Therefore, in the present study using primary female rat hepatocytes we 1) Analyzed the effects of six microsomal enzyme inducers on CYPs expression, and 2) Investigated NDEA participation on CYPs mRNA regulation. The data will provide essential information to whether classes of microsomal enzyme affect rat CYPs mRNA levels at low NDEA doses suggesting potential roles of specific ligand-activated transcription factor pathways.

2. MATERIAL AND METHODS

2.1. Animal Model

This study was conducted in compliance NRC (National Research Council and the "Guide for the Care and Use of Laboratory Animals". Female albine Fischer 344 rats (F-344/DuCrl) from Charles River Laboratories (Germany) between 6 - 8 weeks old were used for the experiments. Light/dark regime was 12/12 h, and standart pelleted rat feed and drinking water were supplied ad libitum.

2.2. Hepatocyte Isolation and Culture

Hepatocytes were prepared according to the two-step collagenase perfusion method [19] with modifications [20]. Fischer 344 female rats were anesthetized with sodium pentobarbital (200 mg/kg) and following hepatic portal vein cannulation, livers were perfused with 200 mL of solution A (NaCl 142 mM, KCl 6.7 mM, HEPES 10 mM) pH 7.4, during 12 - 15 min at 15 mL/min. Livers were subsequently perfused with 200 mL of solution C (9:1 of solution A and $CaCl_2$ 5.7mM), pH 7.4, containing 0.5 mg/mL collagenase (Collagenase Sigma IV, 125 CDU/mg, CAS 9001-12-1) for 20 min at 10 mL/min. Perfused livers were excised and dispersed in 50 mL of solution A and shaken in a closed, sterile container at 37˚C for 10 min. Hepatocyte preparation was filtered through 180 μm nylon filter and centrifuged at 500 rpm, for 10 min, at 4˚C. The wash was repeated and the cells resuspended in MEM eagle Ca^{++} 1.8 mM (Gibco), supplemented with $NaHCO_3$ (26.2 mM), pyruvate 1mM, aspartic acid 0.2 mM and L-serine 0.2 mM. Hepatocytes were counted by hemacytometry, and (2 - 5) × 10^5 cells/mL were added to 60 mm, and (2 - 5) × 10^6 cells/mL were added to 90 mm collagen-coated dishes. Hepatocytes were allowed to attach for 2 hours. Viability, measured by Trypan blue staining, was approximately 85% - 90% and only preparations with viabilities greater than 80% were used in experiments. After attachment, the medium was removed and replaced with fresh MEM eagle Ca^{2+} 1.8 mM (Gibco).

2.3. Incubation of Cells

To investigate the effects of drugs on CYP induction, primary rat hepatocytes were incubated after the first medium changed, for 14 - 16 h with the CYP inducers drug. Ethanol (ETOH), Pyrazole (PYR), and Phenobarbital (PB) were prepared in NaCl 0.9% solution and added directly to cultures to give a final concentration of 0.3%, 300 μM and 1 mM. 3-Methylcholanthrene (3-MC), Streptozotocin (STR) and Pregnenolone 16-α carbonitrile (PNC) were dissolved in ethanol 30% and added to culture medium to give a final concentration of 2 μM, 25 μM and 0.15 μM, respectively.

After 14 - 16 h in the presence or in the absence (W/O group) of CYP inducers, rat hepatocytes were incubated during 3 h, at NDEA final concentrations ranging from 0.21 μg/mL to 105 μg/mL. All experiments were performed in triplicate, using five isogenic animals.

2.4. Cytotoxicity

The studies were performed in triplicate as described by Eckl and Riegler (1987) with some modifications [20]. For determination of the number of apoptotic and ne-

crotic cells, MEM Eagle 0.4 mM was replaced by cold fixative methanol-glacial acetic acid (3:1) for 15 min on the petri dishes, rinsed with distilled water for 2 min and air dried. The fixed cells were stained with DAPI (0.2 pg/ml) dissolved in McIlvaine buffer (Citric acid 0.1M, Na_2HPO_4 0.2M, pH 7.0) for 40 min, washed with McIlvaine buffer for 2 min, briefly rinsed with distilled water and mounted in glycerol. 1000 cells/petri dish (3000 cells per animal/group concentration) were analyzed under the fluorescence microscope (Reichert Univar). The cell death processes are presented as the percentage of cells in 3000 total cells per animal/group concentration) analized. Apoptotic cells were characterized by the occurrence of nuclear condensation, DNA fragmentation, and perinuclear apoptotic bodies, using a fluorescence microscopy (×100 or higher). Necroses were characterized by clumping, increased vacuolation and swelling on the cell membrane.

2.5. RNA Isolation

Total RNA was isolated with TRIzol (Invitrogen, Germany) and treated with DNase I (0.5 Units) (Invitrogen), according to the manufacturer's instructions, and disolved in DEPC water. The concentration of the isolated total RNA was spectrophotometricaly determined at A_{260nm} and diluted in DEPC water at 2 µg/µL. Aliquots of RNA were analyzed by agarose/formadehyde gel electrophoresis to check RNA integrity and stored at –20˚C until further use.

Development of primers for quantitative Real Time PCR Coding sequences for the genes listed in **Table 1**, were obtained from GenBank (http://www.ncbi.nml.nih.gov/GenBank). Target regions within the coding sequences were determined through nucleotide sequence alignment comparisons of target within multiple member gene families using Vector NTI (Informax, Inc., Bethesda, MD). A subfamily-specific region for each CYP was selected as the site of hybridization for either the 5' or 3' CYP PCR primer, and then complementary PCR oligos were screened on the basis of 1) Similar melting temperatures, 2) Similar oligo length and, 3) The prodution of a PCR amplicon with greater than 50% GC content. All primers were submitted to the National Center for Biotechnological Information for nucleotide comparison using the basic logarithmic alignment search tool (BLASTn) to ensure specificity.

2.6. cDNA Synthesis

First-strand cDNA synthesis was performed on the remaining DNase-treated total RNA which was reverse-transcribed using Oligo primers (0.3 ng/µL) and SuperscriptTM III bulk mix (Invitrogen), according to the manufacter's instructions. In addition, duplicate no template control samples were run in identitical conditions.

Table 1. Primer sequences used for Real-Time Quantitative RT-PCR.

Gene/location	Tm ˚C	Primer	Sequence	Amplicon bp
18S RNA (V01270)	67.5	F	AGTCCCTGCCCTT TGTACACACCGC ACCATC-	152
	69.5	R	CAATCGGTAG- TAGCGACGGG	
CYP2A1 (J04187)	68.1	F	TCAATCCTCACTG GCCAC- TATGCTGGACA	90
	69.5	R	CAGAGGGACAC- CAAGAGCAT- GACGCTC	
CYP2B1 (AJ320166)	68	F	CAGCCAGGTGTTT GAGTTCTTCTCTG GG	122
	66.4	R	CCCTGTGCTTCTCC ACAATATGGCCA	
CYP2B2 (J00720)	66.1	F	ACATGTGAACA- GAGATTTCAT- GAGTACA- CATCTCAT	148
	68.3	R	TGTAGACATAG- CACTGAGAC- CATATACA- GAGTCCAT	
CYP2E1 (J02627)	66.8	F	CTCCTCGTCATATC CATCTGGAAGAA- GATCT	127
	63.7	R	TGGTGAAA- GACTTGGGGA- TATCCTTCAA	
CYP3A1 (M10161)	63.9	F	GTTTATGGAAATTC GATGTGGAGTGCC AT	153
	64.4	R	CCCGCCGGTTTGT GAAGACAGAAA	

2.7. Real Time qRT-PCR Analysis

Quantitative RT-PCR reactions were performed using the reagent mix and protocol contained in the BioRad iCycler iQ Real-Time Detection System (BioRad). Reactions were run in duplicate, in a volume of 10 µL, containing 5 µL of the cDNA diluted 1:20 in DEPC water, 4.4 µL of the iQ SYBR Green Supermix and 0.6 µL of the primers mix (forward and reverse primers) at a 3 pmol/µL. The cycle was programmed to 95˚C, 3 min; 40 × (95˚C 1 min, 65˚C 1 min and 72˚C 1 min); 95˚C 1 min; 55˚C 1 min. For the melting curve analysis, it was used a gradient from 55˚C to 95˚C. Real-time PCR data were collected and analyzed on a Rotor-Gene 3000 (Corbett Research). The products were checked by agarose gel electrophoresis and by sequencing.

The endpoint used in the real-time PCR quantification, CT value, is defined as the PCR cycle number that crosses an arbitrarily placed signal threshold. Average CT values from duplicate PCR reactions were normalized to average CT values for housekeeping gene from

Table 2. NDEA cytotoxicity followed administration of classical CYP inducers ETOH (0.3%), PYR (300 µM) and PB (1 mM).

Inducer	Assay conditions	% Survival	% Necrosis	% Apoptosis
	NaCl 0.9%	98.7 ± 0.3	1.1 ± 0.4	0.2 ± 0.1
	0.21 µg/mL	98.2 ± 0.4	1.6 ± 0.3	0.2 ± 0.1
W/O	2.1 µg/mL	97.4 ± 0.5*	2.4 ± 0.5*	0.2 ± 0.1
	21 µg/mL	97.2 ± 0.7*	2.4 ± 0.6*	0.4 ± 0.2
	105 µg/mL	97.2 ± 1.0	2.5 ± 1.0	0.4 ± 0.1*
	NaCl 0.9%	98.1 ± 0.6	1.4 ± 0.4	0.6 ±0.2
	0.21 µg/mL	97.7 ± 0.6	1.6 ± 0.4	0.7 ± 0.1†
ETOH	2.1 µg/mL	97.5 ± 0.6	1.5 ± 0.3	1.0 ± 0.3†
	21 µg/mL	96.6 ± 1.4	2.5 ± 0.7	1.2 ± 0.4†
	105 µg/mL	95.7 ± 1.1	2.6 ± 0.8	1.7 ± 0.2*††
	NaCl 0.9%	98.7 ± 0.5	0.9 ± 0.1	0.4 ± 0.4
	0.21 µg/mL	98.1 ± 0.2	1.2 ± 0.2	0.8 ± 0.0††
PYR	2.1 µg/mL	97.0 ± 0.7	2.1 ± 0.4	0.9 ± 0.3†
	21 µg/mL	95.1 ± 1.0*	3.0 ± 0.2**	2.0 ± 0.8†
	105 µg/mL	95.9 ± 0.4*	3.0 ± 0.1**	1.1 ± 0.2†
	NaCl 0.9%	98.0 ± 0.2	1.3 ± 0.1	0.8 ± 0.1††
	0.21 µg/mL	97.9 ± 0.5	1.5 ± 0.3	0.7 ± 0.2†
PB	2.1 µg/mL	93.8 ± 0.3**††	4.8 ± 0.5*†	1.4 ± 0.2††
	21 µg/mL	93.4 ± 0.4**††	4.7 ± 0.0**†	2.0 ± 0.4*††
	105 µg/mL	92.9 ± 0.4**†	3.6 ± 0.3*	3.6 ± 0.1**††

Mean ± SEM of 3 female Fischer 344 rat livers. Were analysed 3000 cells per animal/group concentration. Statistical analysis, in order to compare the NDEA cytotoxic effect related with the control (NaCl 0.9%) shown significant differences (*$p < 0.05$ and **$p < 0.01$) into the same inducer group. Comparative statistical analysis were made between W/O group and the CYP inducers group (†$p < 0.05$ and ††$p < 0.01$) at same NDEA concentration.

Table 3. NDEA cytotoxicity followed administration of classical CYP inducers ETOH (0.3%), 3-MC (2 µM), STR (25 µM) and PNC (0.15 µM)

Inducer	Assay conditions	% Survival	% Necrosis	% Apoptosis
	NaCl 0.9%	98.1 ± 0.6	1.4 ± 0.4	0.6 ± 0.2
	0.21 µg/mL	97.7 ± 0.6	1.6 ± 0.4	0.7 ± 0.1
ETH	2.1 µg/mL	97.5 ± 0.6	1.5 ± 0.3	1.0 ± 0.3
	21 µg/mL	96.6 ± 1.4	2.5 ± 0.7	1.2 ± 0.4
	105 µg/mL	95.7 ± 1.1	2.6 ± 0.8	1.7 ± 0.2*
	NaCl 0.9%	95.4 ± 0.5	3.8 ± 0.6†	0.9 ± 0.1†
	0.21 µg/mL	93.6 ± 0.7	5.7 ± 0.9†	0.7 ± 0.2†
3-MC	2.1 µg/mL	95.6 ± 0.3	3.3 ± 0.2†	1.2 ± 0.0*
	21 µg/mL	95.9 ± 0.1	2.8 ± 0.3	1.4 ± 0.1*
	105 µg/mL	95.9 ± 0.6	3.1 ± 0.6	1.1 ± 0.0
	NaCl 0.9%	98.8 ± 0.4	0.9 ± 0.2	0.3 ± 0.2
	0.21 µg/mL	98.2 ± 0.0	1.1 ± 0.1	0.8 ± 0.1
STR	2.1 µg/mL	97.1 ± 0.6	2.1 ± 0.4	0.8 ± 0.2
	21 µg/mL	93.7 ± 0.9*†	3.9 ± 1.0	2.5 ± 0.1**
	105 µg/mL	96.2 ± 0.0**	2.8 ± 0.1**	1.0 ± 0.1*
	NaCl 0.9%	95.0 ± 0.1	4.3 ± 0.2†	0.8 ± 0.1†
	0.21 µg/mL	92.9 ± 0.3*	6.5 ± 0.2*††	0.6 ± 0.1††
PNC	2.1 µg/mL	95.2 ± 0.8	3.7 ± 0.9	1.1 ± 0.1
	21 µg/mL	95.8 ± 0.1	3.0 ± 0.1*	1.3 ± 0.0**
	105 µg/mL	95.7 ± 0.5	3.1 ± 0.7	1.2 ± 0.2

Mean ± SEM of 3 female Fischer 344 rat livers. Were analysed 3000 cells per animal/group concentration.Statistical analysis, in order to compare the NDEA cytotoxic effect related with the control (NaCl 0.9%) shown significant differences (*$p < 0.05$ and **$p < 0.01$) into the same inducer group. Comparative statistical analysis were made between ETH group and the CYP inducers group (†$p < 0.05$ and ††$p < 0.01$) at same NDEA concentration.

the same cDNA preparations (Δ CT). The ratio of expression of each CYP gene induced vs. expression of each CYP gene spontaneously induced was calculated as ΔΔCT. The fold induction of each CYP gene is calculated as $2^{-(\Delta\Delta CT)}$ as recommended by Perkin-Elmer. Values were reported as an average of triplicate analyses.

The amount of each gene target in different groups was normalized to an endogenous control (18S ribosomal mRNA).

3. RESULTS

The incubation of the hepatocytes with either each inducer or with different concentrations of NDEA alone did not result in cell viability lower than 90%, as shown in **Tables 2** and **3**.

However, a discrete cytotoxicity was observed when PB, STR and PNC were incubated with hepatocytes in the presence of NDEA at 2.1 - 105 µg/mL, 21 µg/mL, and 0.21 µg/mL, respectively. Most of the cytotoxicity was a result of the increase of the necroses rate. ETOH, PYR and PB increased the rate of apoptoses compared to the control group either with or without different concentrations of NDEA (**Tables 2** and **3**).

The effect of different classical CYP inducers and of different concentrations of NDEA, either alone or together, on the expression of CYP mRNAs in rat hepatocytes is shown in **Figures 1-5**. **Figure 1** shows that STR

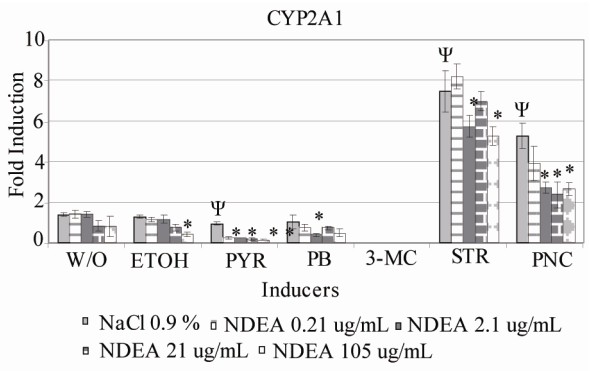

Figure 1. Data has been reported as fold induction of the house keeping gene (18S), for mRNA levels of CYP2A1, under different NDEA concentrations and CYP inducers, measured by real-time RT-PCR.* represents the significant difference ($p < 0.05$) between the NDEA treatment and the control NaCl 0.9%, with the same inducer treatment. Ψ represents the significant difference ($p < 0.05$) at same NDEA concentration (or solvent) between the inducer control group (W/0 for ETOH, PYR and PB treatment, and ETOH for 3-MC, STR and PNC treatment) and the inducer.

and PNC caused an induction (7- and 5-fold, respectively) of CYP2A1 expression, when compared to the solvent treated hepatocytes (W/o group with NaCl 0.9%). NDEA produced a statistically significant decrease of the CYP2A1 induction produced by STR (NDEA con-

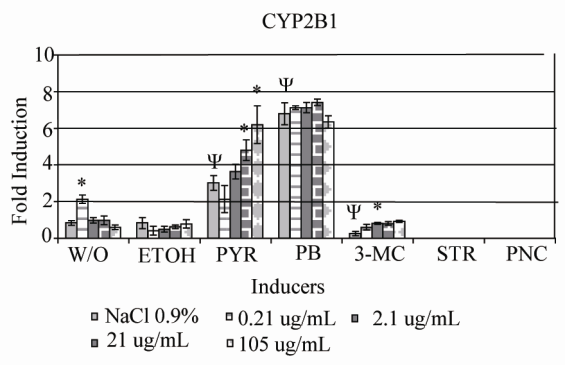

Figure 2. Data has been reported as fold induction of the house keeping gene (18S), for mRNA levels of CYP2B1, under different NDEA concentrations and CYP inducers, measured by real-time RT-PCR.* represents the significant difference ($p < 0.05$) between the NDEA treatment and the control NaCl 0.9%, with the same inducer treatment. Ψ represents the significant difference ($p < 0.05$) at same NDEA concentration (or solvent) between the inducer control group (W/0 for ETOH, PYR and PB treatment, and ETOH for 3-MC treatment) and the inducer. STR and PNC were not performed.

centrations of 2.1 and 105 µg/mL) and by PNC (NDEA concentrations from 2.1 to 105 µg/mL). NDEA also reduced CYP2A1 mRNA levels in the presence of ETOH (NDEA at 105 µg/mL), PYR (NDEA at all doses tested), and PB (NDEA at 2.1 µg/mL).

Figure 2 shows that PYR and PB caused an induction of (3.6- and 8.1-fold, respectively), whereas 3-MC reduced (3.2-fold) CYP2B1 expression. Surprisingly, NDEA (0.21 µg/mL) produced a 2.5-fold induction of CYP2B1. The two highest concentrations of NDEA tested (21 and 105 µg/mL) increased the induction produced by PYR on CYP2B1 levels (1.6 and 2-fold, respectively). When NDEA (at 2.1 µg/mL) was present with 3-MC, it abolished the decrease on CYP2B1 expression produced by 3-MC. CYP2B2 mRNA expression was also induced by PYR and PB (10- and 2.3-fold, respectively), and decreased by 3-MC (3.4-fold), as shown in **Figure 3**. NDEA caused an increase of the CYP2B2 mRNA levels (from 2.8- to 1.5-fold at concentrations ranging from 0.21 to 21 µg/mL). NDEA increased the induction produced by PYR on CYP2B2 expression (1.4 to 2.1-fold for NDEA concentrations ranging from 2.1 to 105 µg/mL), and, at 21 µg/mL, it partially decreased the reduction produced by 3-MC.

CYP2E1 expression was decreased after PYR (2.0-fold), STR (1.8-fold) and PNC (1.6-fold) treatments, as shown in **Figure 4**. Similarly to CYP2B1 and CYP2B2, CYP2E1 was also induced by NDEA, with an inverse relationship between the NDEA dose tested and the fold-induction observed (from 2.6- to 1.6-fold at NDEA concentrations ranging from 0.21 to 21 µg/mL). However,

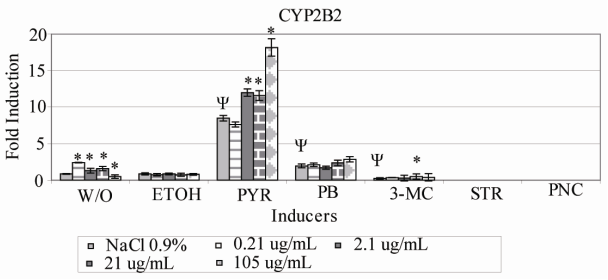

Figure 3. Data has been reported as fold induction of the house keeping gene (18S), for mRNA levels of CYP2B2, under different NDEA concentrations and CYP inducers, measured by real-time RT-PCR. * represents the significant difference ($p < 0.05$) between the NDEA treatment and the control NaCl 0.9%, with the same inducer treatment. Ψ represents the significant difference ($p < 0.05$) at same NDEA concentration (or solvent) between the inducer control group (W/0 for ETOH, PYR and PB treatment, and ETOH for 3-MC treatment) and the inducer. STR and PNC were not performed."

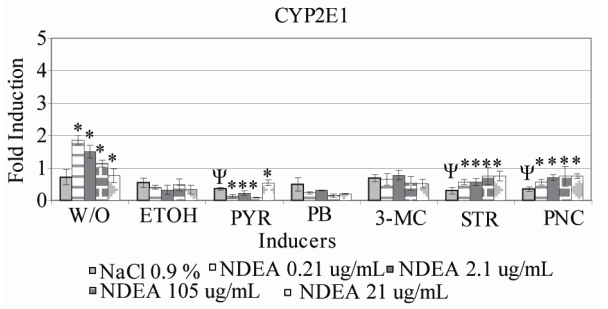

Figure 4. Data has been reported as fold induction of the house keeping gene (18S), for mRNA levels of CYP2E1, under different NDEA concentrations and CYP inducers, measured by real-time RT-PCR. *Represents the significant difference ($p < 0.05$) between the NDEA treatment and the control NaCl 0.9%, with the same inducer treatment. Ψ Represents the significant difference ($p < 0.05$) at same NDEA concentration (or solvent) between the inducer control group (W/0 for ETOH, PYR and PB treatment, and ETOH for 3-MC, STR and PNC treatment) and the inducer.

different from CYP2B1 and CYP2B2, CYP2E1 expression was reduced when PB and PYR were incubated with hepatocytes in the presence of NDEA. NDEA abolished the decrease in CYP2E1 expression produced by either STR or PNC.

CYP3A1 expression was induced by PNC (47-fold), and decreased by ETOH (2.6-fold). NDEA (2.1 mg/ml) also induced CYP3A1 mRNA. However, NDEA decreased the induction produced by PNC (4.8- to 2.7-fold, for NDEA concentrations ranging from 2.1 to 105 µg/mL) and it increased the reduction produced by ETOH on CYP3A1 expression (1.8-fold at 105 µg/mL). There was also a reduction of CYP3A1 when STR was incubated with the three highest doses of NDEA tested (from 5.5- to 3.1-fold for NDEA concentrations ranging

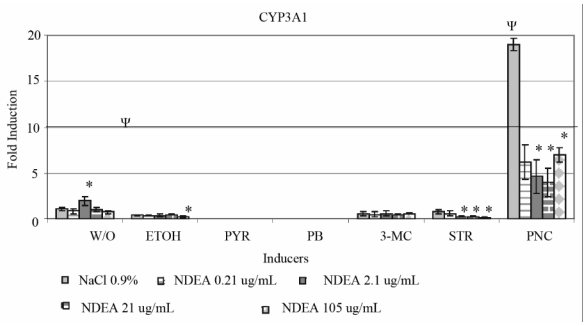

Figure 5. Data has been reported as fold induction of the house keeping gene (18S), for mRNA levels of CYP3A1, under different NDEA concentrations and CYP inducers, measured by real-time RT-PCR. *Represents the significant difference ($p < 0.05$) between the NDEA treatment and the control NaCl 0.9%, with the same inducer treatment. Ψ Represents the significant difference ($p < 0.05$) at same NDEA concentration (or solvent) between the inducer control group (W/0 for ETOH treatment, and ETOH for 3-MC, STR and PNC treatment) and the inducer. PYR and PB were not performed."

from 2.1 to 105 µg/mL) (**Figure 5**)

4. DISCUSSION

Until last decades, NDEA was considered a weak mutagen in classical genotoxicity assays [21-23]. Aiub, *et al.* [4,24-25], standardized some genotoxicity assay (Ames and SOS chromotest) conditions in order to show the real genotoxicity potential of NDEA. Using more sensitive strains for the Ames test, positive results were detected for NDEA at doses between 1.01 ng/mL and 50.64 ng/mL, when 4% metabolic activation mixture (S9 mix) was present [16]. Although short-term in vitro tests for genotoxicity, play an important role in the initial screening of drugs in order to check their potential to induce mutagenic/carcinogenic effects, it is very important to include assays that use mammalian cells, such as primary cultures of rat hepatocytes, when these systems maintain metabolic competence for the bioactivation of xenobiotics by CYP enzymes. The genes encoding these enzymes are either expressed constitutively or are induced by various chemicals [26]. Hepatocytes, as the cells that express higher CYP levels, are the most suitable model to investigate CYP induction and their relation with drug metabolism.

In the present work we show that the administration of certain chemicals results in an up or down regulation of the transcription of different forms of CYP genes.

Human and rodent CYP2E1, 2A and 3A play important roles in the metabolic activation of carcinogenic *N*-nitrosamines [27-29]. From the present data we can suggest the necessity of CYP2A1, 2B1, 2B2, 2E1 and 3A1 induction in NDEA-treated hepatocyte rats and also recommend a combined treatment for preparation of the S9 fraction in short-term genotoxicity assays.

The *N*-nitroso compound was able to induce at very low doses, CYP2A1 (0.21 µg/mL), CYP2B2 (0.21 - 21 µg/mL), CYP2E1 (0.21 - 105 µg/mL) and CYP3A1 (2.1 µg/mL), in the absence of inducers (W/O group). Otherwise for some of the tested inducers, in the presence of NDEA, a dose-dependent inhibition of CYP2A1 mRNA (for PYR, STR and PNC treatment, **Figure 1**), CYP2B2 mRNA (for ETOH, **Figure 3**), CYP2E1 mRNA (ETOH, PYR, PB, STR and PNC, **Figure 4**), and CYP3A1 mRNA (for ETOH and STR treatment, **Figure 5**) was observed. It is known that CYP enzymes exhibit liver-specific expression driven by transcriptional factors [30]. One possibility is that NDEA metabolites or NDEA by itself, are able to interact negatively on the orphan receptors (ear-2 and ear-3) or in concert with other transcriptional factors (HNF-4 and ERα). Miyazaki et al. (2005) [31] and Weymarn, *et al.* (2005) [32] have also shown that the pre-treatment of female A/J mice with 8-methoxypsoralen inhibits the CYP2A family.

Although 3-MC and PB are known to induce CYP2A1 protein [33], the effect of NDEA on CYP2A induction by PB did not show significant effects.

Concerning NDEA's effects as CYP inducer, fold induction of CYP2B1 and CYP2B2 present similar profile curves although with different intensities. Both CYPs were strongly induced by PYR (CYP2B1 at 21 - 105 µg/mL NDEA concentration; CYP2B2 at 2.1 - 105 µg/mL) suggesting a synergistic effect on mRNA CYP expression signaling (**Figures 2** and **3**). An increase in the expression of CYP2B1 and CYP2B2 mRNA levels in the presence of PYR can lead to an increase in the NDEA metabolites and consequently on the DNA lesions. The data herein, show for the first time, the induction of a CYP2B1 and CYP2B2 by NDEA and provide basis for a mechanism by which NDEA could modulate biological processes.

3-MC and PB suppress hepatic levels of CYP2E1 protein and metabolic activity in rats [34-36]. We observed a decrease on the CYP2E1 mRNA levels in the presence of NDEA for ETH, PYR and PB inducer treatments. The cultures CYP pre-induced by 3-MC did not present significantly changes compared with the control group (ETOH).

CYP2E1 has a remarkable capacity to activate low molecular weight *N*-nitrosamines such as NDEA [28]. Mori, *et al.* (2001) observed that the inducer 4-methylpyrazole markedly inhibit DMN activity and showed a higher induction of CYP2B1/2, rather than CYP2E1 [37]. This is in agreement with our findings for CYP2B1, CYP2B2 mRNA, while for CYP2E1 mRNA is inhibited, suggesting that NDEA activates CYP2B gene expression

at a pretranslational level.

As described by Honkakoski and Negishi (2000) several CYPs degrade ligands that activate the NR responsible for the regulation of this specific CYP form, creating a direct feedback loop [38]. In this way, the observed induction of CYP2B1 and CYP2B2 by NDEA and PYR, can act inhibiting CYP2A1 and CYP2E1.

In vitro assays with rodents, generally the only cultured cells in which CYP2B genes respond normally to PB treatment, are primary hepatocytes [39,40]. The mechanism for this is based on the PB response unit, that confers PB inducibility on heterologous promoters presenting transcriptional enhancer properties [41]. Comparing the effects on mRNA levels on **Figures 4** and **5**, for PB treatment and concerning the fact that PB enhances the mRNA levels of CYP2B1 > CYP2B2, independently of NDEA concentrations, we can suggest that NDEA or it metabolites, can enhance the mRNA levels of CYP2B1 by others unknown mechanisms.

Comparing the mRNA levels of CYP2B1, CYP2B2, CYP2E1 and CYP3A1 from ETOH-treated hepatocytes with the cells without inducer, we can observe a repression in their expressions (**Figures 2-5**). Ethanol has been known as a substrate of CYP2E1 [42]. The constitutive level of hepatic CYP2E1 is regulated transcriptionally by liver-enriched transcription factors, especially by HNF-1 [43].

Regarding the CYPs levels of mRNA in culture, it has been shown that rat hepatocyte in the presence of 3-MC (2 μM) and PB (1 mM) after second day in culture need supplementation [44]. The cells were submitted to inducer drugs after 6 hours platting and kept for 16 - 18 hours until NDEA added. Then, these drugs (inducers and NDEA) remained for 3 hours in the medium.

5. CONCLUSIONS

We have demonstrated that NDEA has a multifunctional action with wide spectrum induction of phase I enzymes and alone or in combination with different inducers would be a pertinent inducer for metabolic enzymes in in vitro bioassays. This pattern provides a general outline of the induction/repression effects of NDEA and the inducers used, leading to further mechanistic studies.

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