Structural influence of isothiocyanates on expression of cytochrome P450, phase II enzymes, and activation of Nrf2 in primary rat hepatocytes

M. La Marca, P. Beffy, C. Della Croce, P.G. Gervas, R. Iori, E. Puccinelli, V. Longo

Abstract

Primary cultures of rat hepatocytes were used to investigate whether and how eight isothiocyanates (ITCs) with different chemical structures (the aromatic benzyl, 4-hydroxybenzyl, phenethyl isothiocyanates and the aliphatic allyl, napin, iberin, raphasatin isothiocyanates and sulforaphane) derived from hydrolyzed glucosinolates, were able to modulate cytochrome P450 (CYP) and antioxidant/detoxifying enzymes and to activate the Nrf2 transcription factor. The aromatic ITCs at 40 μM markedly increased the transcription of CYP1A1 and 1A2 mRNA and increased the associated ethoxyresorufin O-deethylase (EROD) activity after 24 h of treatment. By contrast, the aliphatic ITCs (40 μM) decreased CYP1A1 and 1A2 transcription and induced catalase activity. The same treatment also caused a striking and similar transcriptional repression of CYP3A2, and the corresponding benzyloxyquinoline debenzylase activity in response to all the ITCs tested. In the same culture conditions, most of the antioxidant/detoxifying enzymes were significantly up-regulated by 40 μM ITCs. In particular, NAD(P)H:quinone oxidoreductase and heme oxygenase-1 were induced, although to different levels, at transcriptional, protein and/or activity levels by all the ITCs. However, glutathione S-transferase activity was not induced by the allyl, benzyl, and 4-hydroxybenzyl ITCs, glutathione reductase activity was not induced by benzyl, and 4-hydroxybenzyl ITCs and catalase activity was not induced by allyl ITC. As for the Nrf2 transcription factor, a partial translocation of its protein from the cytosol to the nucleus was revealed by immunoblotting after 1 h of treatment for all the ITCs tested. The ability of ITCs to induce the antioxidant and phase II enzymes did not appear to be affected by their hydrophilicity or other structural factors. Taken together, these results show that these ITCs are effective inducers of ARE/Nrf2-regulated antioxidant/detoxifying genes and have the potential to inhibit, at least in rat liver, the bioactivation of carcinogens dependent on CYP3A2 catalysis.

1. Introduction

A variety of studies have consistently shown that phytochemicals present in foods and food products play a protective role in the etiology of various diseases (Xiang et al., 2009). Over the past 20 years, glucosinolates (GLs) and their hydrolysis products, isothiocyanates, have received much attention by scientists thanks to their anticancer and antioxidant properties (Lam et al., 2009; Talalay, 1994). Most of the dietary isothiocyanates absorbed by mammals from ingested plant material are formed by the action of myrosinase from gastrointestinal tract bacteria. ITCs are abundant in cruciferous vegetables such as broccoli, watercress, Brussels sprouts, cabbage and cauliflower. Over 120 different GLs have been identified, and they have been classified into three main groups: (1) the aliphatic group characterized by an alkyl or alkenyl side-chain (e.g. sinigrin), (2) the aromatic group (e.g. glucoraphanin) (Xiang et al., 2009). GLs are normally found in the cytoplasm of plant tissues where they have little bioactivity. When the tissue is ruptured by chewing, preparation for cooking, heating or insect attack, the glucosinolates are released and can interact with myrosinase, an enzyme present in the plant cell wall, and hydrolysis products are formed. At neutral pH, the GLs are mainly hydrolyzed to stable ITCs and, in small amounts, to other compounds such as nitriles and thiocyanates (Zhang and Talalay, 1994). Most of the dietary isothiocyanates absorbed by mammals from ingested plant material are formed by the action of myrosinase from gastrointestinal tract bacteria.

It is slowly emerging that the mechanisms underlying the cancer chemopreventive effects of ITCs are multiple (Conaway et al., 2002; Juge et al., 2007; Cheung and Kong, 2010; Zhang, 2010) and include the induction of apoptotic pathways, the inhibition of angiogenesis, their anti-inflammatory properties, and the modulation of phase I and phase II biotransformation enzymes. These
enzymes catalyze a variety of hydrolytic, oxidative, and reductive reactions (phase I) and conjugation reactions (phase II). The ability of ITCs to modulate the biotransformation enzymes is thought to be connected to their ability to inhibit the catalytic activity of a number of cytochrome P450 (CYP) enzymes, which activate certain carcinogens, and is also thought to be connected to their ability to induce the expression of some antioxidant and phase II enzymes (Hayes et al., 2008).

ITCs such as phenylethyl isothiocyanate (PEITC) and other aromatic isothiocyanates, when administered to mice or rats, inhibit 4-((methyl)nitrosamino)-1-(3-pyridyl)-1-butanone-induced tumor formation as well as some cytochrome P450s (CYP) including CYP1A1, 1A2, 2B1 and 2E1 (Yang et al., 1994). In vitro experiments using rat liver microsomes have demonstrated that aromatic ITCs are strong inhibitors of EROD and PROD (dependent on 1A1/2 and 2B1, respectively), but the alkyl ITCs such as sulforaphane (SFN) and allyl isothiocyanate (AITC) are weak inhibitors of these CYP isoforms (Conaway et al., 1996).

Goosen et al. (2001) have also described a mechanism-based inhibition of CYP1A1, 1A2, 2B1 and 2E1 but not 2C9, 2D6 and 3A2 by benzyl isothiocyanate (BITC) in rat liver microsomes. However, the administration of ITCs to rodents may provoke either an increase or decrease of specific CYP activity and tumorogenesis, depending on which ITC is used and on the treatment regimen (Zhang and Talalay, 1994). For example, the chronic administration of PEITC was found to increase the levels of both CYP2B1 and 2E1 as well as the carcinogenic toxicity (Smith et al., 1993).

From the previous example, it is clear that the results of CYP inhibition obtained in vitro with microsomes or recombinant enzymes might differ to those obtained in vivo. It is possible that the use of living cells (hepatocytes or liver slices) might better reflect what occurs in vivo. However, few studies have used primary hepatocytes to evaluate the effects of ITCs on phase I and II enzymes. Studies conducted in primary rat hepatocytes show the potential of the aliphatic SFN to markedly inhibit EROD and PROD activity (markers of CYP1A1/1A2 and 2B1, respectively) (Mahèo et al., 1997), whereas different effects were obtained with rat liver slices treated with the aliphatic SFN and erucin (Hanlon et al., 2008). In human hepatocytes, SFN greatly decreased the expression of the CYP1A1, 1A2 and CYP3A4 genes, whereas the aryl PEITC strongly induced the expression of the CYP1A1 and 1A2 genes (Gross-Steinmeyer et al., 2010).

As far as phase II is concerned, it was proposed that ITCs operate by activating the nuclear factor E2-related protein (Nrf2) (Fahey et al., 1997). Nrf2 is a basic-leucine zipper transcription factor which, under basal conditions, is present in an inactive form in the cytoplasm, bound to the Kelch-like ECH-associated protein 1 (Keap1) (Itoh et al., 1999). Various agents, such as ROS or weak electrophiles (e.g. ITCs), can alter the Keap1-Nrf2 protein complex and liberate Nrf2 by phosphorylating or alkylating one or more of the 27 cysteine residues of Keap1. Nrf2 is then free to migrate into the nucleus. Once free from Keap1, Nrf2 dimerizes with small Maf protein and binds to antioxidant responsive element (ARE) sites in the promoter of antioxidant and phase II genes, thereby inducing the transcription of these genes.

Many authors have studied the ability of a wide range of ITCs to induce the ARE/Nrf2 regulated enzymes. In particular, a great deal of attention has been to the NAD(P)H:quinone oxidoreductase (NQO1) – known as the most sensitive ARE/Nrf2 responsive enzyme. It has been studied in various hepatoma cell lines (Postner et al., 1994; Tawflq et al., 1995; Ye and Zhang, 2001; Vermeulen et al., 2006). Some authors have also verified what causes the activation of Nrf2 signaling pathway (Jeong et al., 2005; Keum et al., 2008; Prawn et al., 2008). However, studies of ITCs in rat and human hepatocytes or liver slices are limited to SFN, PEITC and erucin and only the induction of NQO1 and other enzymes were evaluated but not the activation of Nrf2 (Mahèo et al., 1997; Payen et al., 2001; Hanlon et al., 2009; Gross-Steinmeyer et al., 2010).

The main aim of our work was to investigate whether various GL hydrolysis-derived ITCs, with specific chemical structures, were differently able to modulate phase I and phase II enzymes at catalytic and/or transcriptional levels. For this, we used sandwich cultures of primary rat hepatocytes, a unique in vitro system that maintains specific hepatic cytomorphology as well as the function of their drug metabolism, deposition and toxicity, closely resembling the in vivo setting (Swift et al., 2010). GL hydrolysis-derived aromatic ITCs (benzyl, 4-hydroxybenzyl and phenethyl isothiocyanates), alkyl aliphatic ITCs (allyl and napin isothiocyanates) and aliphatic ITCs with an additional sulfur atom (ibiberin, raphasatin and sulforaphane isothiocyanates), were assayed (Table 1). Instead of synthetic or pure ITCs we chose to study different GL hydrolysis products in order to better simulate in vivo conditions, where the biotransformation of glucosinolates to isothiocyanates occurs in the intestine by microbial myrosinase.

## 2. Materials and methods

### 2.1. Chemicals

Collagenase; dexamethasone; insulin; glucagon; penicillin/streptomycin; ampicillin/kanamycin; fetal bovine serum. The cells were plated at a density of 4.5 × 10⁶ cells/ml on 100 mm cell culture dish pre-coated with 3 ml of collagen (type I) solution.

### 2.2. Preparation of glucosinolates

The glucosinolates were isolated from different plant sources using an improved purification method (Visentin et al., 1992) and the purity of GLs as determined by HPLC and NMR spectrometry, was in the range of 95–99% (Razis et al., 2008).

### 2.3. Primary rat hepatocytes isolation, culture and treatments

Hepatocytes were isolated from 200 to 300 g Wistar male rats with free access to drinking water and food and on a 12 h light/dark cycle. Rats were anesthetized with 40 mg/kg ketamine (Zoletil™) and the liver was perfused as described previously (De Smet et al., 1998). After filtration and centrifugation, the cell viability was determined by trypan blue exclusion. The cells were dispersed in Williams E medium containing 39 mg/ml dexamethasone, 0.5 U/ml insulin, 0.007 µg/ml glucagon, 5 µg/ml penicillin and streptomycin, 5 µg/ml ampicillin and kanamycin and 10% fetal bovine serum. The cells were plated at a density of 4.5 × 10⁶ cells/ml on 100 mm cell culture dish pre-coated with 3 ml of collagen (type I) solution (1 mg/ml). The cultures were maintained at 37 °C in 5% CO₂ in a humidifier incubator. After 5 h, the medium was replaced with serum-free Williams E medium supplemented with 2% BSA, 7.5 µg/ml hydrocortisone 21-hemisuccinate sodium salt

## Table 1

<table>
<thead>
<tr>
<th>Glucosinolates</th>
<th>Isothiocyanates (ITCs)</th>
<th>–R groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucoraphanin</td>
<td>Sulforaphane (SFN)</td>
<td>CH₃S(O)CH₂CH₂CH₂⁻</td>
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<tr>
<td>Glucoiberin</td>
<td>Ibrin isothiocyanate</td>
<td>CH₃S(O)CH₂CH₂CH₂⁻</td>
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<tr>
<td>Glucoraphasin</td>
<td>Raphasatin isothiocyanate (RITC)</td>
<td>CH₃S(O)CH₂CH₂CH₂⁻</td>
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<tr>
<td>Glucoraphacin</td>
<td>Napin isothiocyanate</td>
<td>CH₂=CHCH₂CH₂⁻</td>
</tr>
<tr>
<td>Sinigrin</td>
<td>Allyl isothiocyanate</td>
<td>CH₂=CHCH₂⁻</td>
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<td>Gluc实在turnin</td>
<td>Phenethyl isothiocyanate (PEITC)</td>
<td>CH₃S(O)CH₂CH₂CH₂⁻</td>
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<tr>
<td>Glucotropeolin</td>
<td>Benzyl isothiocyanate (BITC)</td>
<td>CH₃S(O)CH₂CH₂CH₂⁻</td>
</tr>
<tr>
<td>Glucosinalbin</td>
<td>4-Hydroxybenzyl isothiocyanate (BITC)</td>
<td>CH₃S(O)CH₂CH₂CH₂⁻</td>
</tr>
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and EGF 20 ng/ml. Cultures were maintained in this medium at 37 °C and 5% CO₂ for 24 h. After this period, the medium was removed and a second layer of type I collagen was added to create a collagen-gel sandwich culture (Beken et al., 1998) and after 45 min serum-free Williams E medium was added again. The cells were maintained for additional 24 h before treatments. Glucosinolates (glucoiberin, sinigrin, glucoraphanin) were incubated at 37 °C for 15 min at pH 7.0 with myrosinase to permit a complete hydrolysis that originates stable ITCs (hereafter named ITC solutions) as the only GLs hydrolysis product (Leoni et al., 2000). Cell experiments were performed with different concentrations (20–60 μM) of isothiocyanate solutions for 1, 4, 24 h. At 20–40 μM concentrations, unlike 60 μM, ITCs solutions produced no adverse cytotoxic effects over the exposure periods compared with the vehicle-only controls, as measured by lactate dehydrogenase assay.

2.4. Enzymatic activities

After 24 h the end of treatment, the medium was removed and a collagenase solution was added. After 30 min, recovered cells were centrifuged (400g) for 3 min at 4 °C. The cell pellet was sonicated and used for the microbial preparation (Jagow et al., 1965). Total protein concentration was determined by the method of Lowry et al. (1951). NAD(P)/H-quinone oxidoreductase (NQO1) activity was measured by the method of Benson et al. (1980). Glutathione-S-transferase (GST) activity was quantified as previously described by Habig et al. (1974), using 1-chloro-2,4-dinitrobenzene as substrate. The GSTG reduction activity was measured monitoring the consumption of NADPH for 1 min at 340 nm while the catalase activity was monitored following the H₂O₂ decomposition at 240 nm, as described by Cao and Li (2002). Heme oxygenase-1 (HO-1) activity was determined by the method of Naughton et al. (2002). Lactate dehydrogenase activity was assayed as previously described (Dekker and Lohmann-Matthes, 1988).

In general, the cytochrome P450-dependent enzymatic assays with the hepatocyte microsomes were carried out at 37 °C for 30 min in a 1 ml reaction mixture as previously reported (Amato et al., 1996). The dealkylation activities of ethoxyresorufin-0-deethylase (EROD), ethoxy- and benzoxycoumarin-0-deethylase (ECOD), and benzyloxyquinoline debenzylase (BQD) were measured by monitoring fluorimetrically the formation of the corresponding hydroxyl products as previously reported (Burke and Mayer, 1974; Atio, 1978; Streffer et al., 2000).

2.5. RNA extraction and cDNA synthesis

Total cellular RNA was extracted from primary rat hepatocytes 4 h after ITC treatment, using the RNeasy Mini Kit (Qiagen, Valencia, CA), following the supplied protocol. RNA was quantified using NanoDrop (Celbio, MI, Italy); its purity and integrity were evaluated by checking the absorbance ratio at 260–280 nm and assessing the sharpness of 18S and 28S ribosomal RNA bands on agarose gel stained with ethidium bromide. Genomic DNA elimination and reverse transcription of total RNA were performed using QuantiTect Reverse Transcription Kit (Qiagen).

2.6. RT-PCR

Two microliters of cDNA were added to a PCR Master Mix (GoTaq Green Master Mix, Promega, Madison, WI) for the amplification reaction (various cycles) performed using for each transcript 400 nM of forward–reverse primers for heme oxygenase-1 (GenBank Accession No. NM_012580.2, NQO1 (GenBank Accession No. NM_017000.3), CYP1A1 (GenBank Accession No. NM_012541.3), CYP3A2 (GenBank Accession No. NM_153312.2), β-actin, as housekeeping gene, (GenBank Accession No. NM_031144.2) and the annealing temperature indicated in Table 2. The β-actin controls originate from the same lane of the agarose gel of samples. The DNA fragments were separated on ethidium bromide-stained 1% agarose gel and visualized by transillumination with ultraviolet light. Bands obtained from five independent rat experiments were quantified by an Image J software. The results have been normalized to β-actin levels and are expressed as percentages of control. Results are reported as means ± SD of cells from five independent experiments using five rats.

2.7. Preparation of nuclear fractions

Nuclear and cytosolic extracts were prepared by previously established methods (Balogun et al., 2003). Briefly, hepatocytes were washed twice with 1× PBS. Cells were then harvested in 1 ml of PBS and centrifuged at 800g for 3 min at 4 °C. The pellet was carefully resuspended in 200 μl of cold hypotonic buffer, consisting of 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 μM dithiothreitol and complete protease inhibitor cocktail (Sigma antiprotease cocktail P8340). After addition of NP40 to a final concentration of 0.3%, the cells were vortexed and centrifuged at 800g for 3 min at 4 °C. The resulting nuclear pellet was resuspended in 30 μl of cold nuclear extraction buffer (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 μM dithiothreitol, 25% glycerol and protease inhibitors) and incubated on ice for 30 min. The nuclear extract was finally centrifuged at 15000g for 15 min at 4 °C. The supernatant containing nuclei proteins was aliquoted and stored at −30 °C.

2.8. Immunoblot analysis

Nuclear and microsomal proteins from primary rat hepatocytes were separated according to Laemmli (1970) on SDS-10% (w/v) 1.0 mm thick polyacrylamide gels and then electrophoretically transferred onto nitrocellulose membranes following the method of Towbin et al. (1979). Antibodies used were anti-Nrf2 (1: 1000, sc13032, Santa Cruz Biotecnology, Heidelberg, Germany), anti heme oxygenase-1 (1:1000, sc-10789, Santa Cruz Biotechnology, Heidelberg, Germany), β-actin (1:1000, sc-130657, Santa Cruz Biotechnology, Heidelberg, Germany) and goat anti-rabbit (1:2000 or 1:5000). Immunoreactive proteins were visualized with a chemiluminescence reaction Kit (EuroClone, MI, Italy) and bands obtained from five independent rat experiments were electronically scanned and quantified by an Image J software.

2.9. Statistical analysis

Results are reported as means ± SD. Statistical significance was determined by Student’s t-test for comparison between control and treated groups. p Value < 0.05 was considered to be significant.

3. Results

3.1. The effect of ITC solutions on cytochrome P450

To study the modulation of some CYPs, cells were treated for 24 h with various solutions, each containing one of the following isothiocyanates: AIFC, NITC, PEITC, RITC, HB1T, B1T, IB1T, SFN produced by the hydrolysis of 20–40 μM GLs. The following mono-oxygenase activities were evaluated: ethoxyresorufin-0-deethylase (EROD), marker of CYP3A1/1A2 (Burke and Mayer, 1974), ethoxy- and benzoxycoumarin-0-deethylase (ECOD), marker of many CYPs, the most noteworthy being CYP1A1/2, 2A, 2B, 2C and 2E1 (Funae and Imaoka, 1993), and benzyloxyquinoline debenzylase (BQD), marker of CYP3As (Streffer et al., 2000). In Fig. 1A–C, only the most effective results, obtained with the 40 μM treatments, are depicted. Using 20 μM ITCs, similar enzymatic profiles with lower values were observed (data not shown). As illustrated in Fig. 1A, EROD activity, selective for the CYP1A1/1A2, was significantly inhibited only by the aliphatic ITCs SFN and IB1T. Conversely, the aromatic PEITC, HB1T and B1T increased EROD activity to 178%, 225%, 233% of the control level, respectively. Also ECOD activity, responsive to the catalysis of CYP1A1/1A2, was significantly induced by the same aromatic ITCs, but its inhibition by the aliphatic ITCs was not observed. Surprisingly, BQD activity
was reduced to about 50% of control by all the isothiocyanates tested.

To examine the effect of ITC solutions on CYPs at the transcriptional level, semi-quantitative RT-PCR was performed using the primer pairs shown in Table 2. PCR analysis with a different number of amplification cycles allowed us to compare the levels of CYP1A1, CYP1A2 and CYP3A2 mRNA in rat hepatocytes treated for 4 h with two different concentrations (20 and 40 μM) of ITCs...
solutions (Figs. 2 and 3). In agreement with the EROD activity findings, only treatment with the aromatic ITCs (PEITC, HBITC, BITC) increased both CYP1A1 and CYP1A2 transcripts (Fig. 2). Treatment with AITC, NITC, RITC, IBITC, SFN did not affect CYP1A1 or CYP1A2 expression (data not shown).

In Fig. 3, CYP3A2 mRNA expression is shown. CYP3A2, belonging to the CYP3A subfamily, is highly expressed in the untreated rat liver (Mahnke et al., 1997). Its expression markedly decreased compared to the control level by all the ITCs in a dose dependent manner.

3.2. Effect of ITC solutions on antioxidant and phase II enzyme activity

In the microsomes or cytosol of control hepatocytes and hepatocytes treated for 24 h with 40 \( \mu M \) ITC solutions, the activity of NQO1, HO-1, GSSG reductase, GST, which is regulated by Nrf2 (Juge et al., 2007) and catalase, was determined (Fig. 4A–E).

As expected, in primary rat hepatocytes all the tested compounds induced by about 2- to 3.5-fold NQO1 activity, which was the most sensitive enzyme (Fig. 4A). The greatest enhancements in NQO1 activity of about 3- to 3.5-fold with respect to the control value (52 ± 7.2 nmol/min/mg prot), were observed after treatment with the aromatic PEITC and the aliphatic NITC and RITC, whereas the lowest enhancements were observed in response to the aromatic HBITC and aliphatic AITC treatments, proving that there is no evident relationship between ITCs structure and activity. HO-1 activity (Fig. 4B) was significantly induced by similar amounts (about 1.5- to 2-fold of control value: 17.5 ± 6.1 pmol/min/mg prot) by all the ITCs solutions. GST activity was significantly induced (about 1.3–1.5 of control value: 256 ± 9.3 nmol/min/mg prot) by the NITC, PEITC, RITC, IBITC and SFN but not by the aliphatic AITC and the aromatic HBHTC and BITC (Fig. 4C). We observed a weak though significant increase in glutathione reductase activity (about 1.3- to 1.5-fold of control value: 113 ± 8.1 nmol/min/mg prot) (Fig. 4D), only following treatment with AITC, NITC, PEITC, BITC, IBITC and SFN. RITC and HBITC failed to induce glutathione reductase activity. As for catalase (Fig. 4E), all the ITCs but AITC were able to induce its activity (about 1.5- to 2.5-fold of the control value: 167 ± 6.1 nmol/min/mg prot).

The NQO1 and HO-1 enzymes were chosen for additional analysis at the transcriptional level to further investigate their responsiveness to ITC treatments. The expression of their transcripts were analyzed by RT-PCR in primary rat hepatocytes, using the sets of

![Fig. 3.](image)

![Fig. 4.](image)
primers listed in Table 2. Cells were treated with two concentrations (20 and 40 μM) of ITC solutions for 4 h. As shown in Fig. 5, all the ITC treatments increased the expression of NQO1 and HO-1 genes with respect to the control levels, although to different extents.

The effect of ITC solutions on heme oxygenase-1 was also assessed at the protein level by Western blot analysis. In Fig. 6 it is shown a representative immunoblot detected by chemiluminescence with 5 min exposure using microsomes of five independent experiments. In microsomes from all hepatocytes treated with 40 μM ITCs, the anti-rat HO-1 antibodies revealed a significant increase in intensity of a immunoreactive protein band of about 1.5- to 2.5-fold with respect to the control, in agreement with results from the enzymatic activity assays.

3.3. Effect of ITC solutions on Nrf2

To verify whether the Nrf2 pathway was activated in primary rat hepatocytes treated for 1 h with 40 μM ITC solutions, we analyzed by Western blot cytosolic and nuclear fractions prepared from control and ITCs treated-cells (Fig. 7). A strong protein band in control cytosols was revealed by an anti-rat Nrf2 antibody,

Fig. 5. A representative RT-PCR analysis of NAD(P)H:quinone oxidoreductase (NQO1), heme oxygenase-1 (HO-1) genes performed with 25, 27 and 29 cycles in primary rat hepatocytes of control (CTR) and treated 20 and 40 μM with ITC solutions. PCR products were separated by electrophoresis on agarose gels and stained with ethidium bromide. Quantitative representation of the RT-PCR analysis is reported below. The results have been normalized to β-actin levels and are expressed as percentages of control. Mean ± SE of cells from five independent experiments using five rats. * Significantly different from controls by Student's t-test, p < 0.001.

Fig. 6. Western blot analysis of heme oxygenase-1 protein in microsomes (50 μg) of control (CTR) cells and cells treated for 24 h with 40 μM ITC solutions. Microsomal samples were subjected to SDS–PAGE, electrophoretically transferred to a nitrocellulose membrane, and probed with polyclonal antibodies raised against rat HO-1. Densitometric analysis of the Western blot data are shown on the right side. The results have been normalized to β-actin levels and are expressed as percentages of control. Mean ± SE of cells from five independent experiments using five rats. ** Significantly different from controls by Student's t-test, p < 0.01. *** p < 0.001.
which showed a clear trend to diminish in the cytosol of treated cells (data not shown). This protein band was faint or not visible in the control nuclei but was induced (to different levels depending on the particular ITC used), in the nuclear extracts of all the ITCs treated hepatocytes.

4. Discussion

In the present study, we investigated whether eight ITCs with different chemical structures (the aromatic PEITC, BITC, HBITC, the aliphatic AITC, NITC and the aliphatic with an additional sulfur atom, SFN, IBITC, RITC) were able to modulate CYP isoforms, antioxidant enzymes and phase II enzymes, or activate Nrf2 at the transcriptional and/or catalytic and protein levels in primary rat hepatocytes. Liver parenchymal cells are a widely accepted in vitro model to evaluate mechanisms of drug metabolism. Primary hepatocytes have the necessary intracellular machinery and physiological concentrations of cofactors for both phase I and phase II reactions, as well as the complete array of hepatic transport proteins and nuclear receptors, which allow for proper regulation and induction of enzymes and transport proteins (Swift et al., 2010).

First, we examined the cytotoxic effect of the ITC solutions up to 60 μM in primary rat hepatocytes. The aromatic ITCs proved cytotoxic at the highest concentration used, as assessed by the lactate dehydrogenase activity. The toxic effects of the ITC solutions were compared to the same solutions at lower concentrations (20 and 40 μM) that are not toxic and are similar to those described when pure SFN was tested in primary rat hepatocytes (Payen et al., 2001) and when other ITCs were assayed in Hepa-1c1c cells (Tawfiq et al., 1995). Thus, in our culture conditions, the ITC solutions did not cause any particular over toxicity. When assayed in the hepatic cells at 40 μM for 24 h, only the aliphatic SFN and IBITC caused a significant inhibition of the microsomal EROD activity (a selective marker for CYP1A1 and 1A2) but not ECOD activity (a marker for many CYPs including CYP1A1 and 1A2). By contrast, the treatment with the aromatic ITCs (PEITC, BITC and HBITC) significantly increased both EROD and ECOD activity and the expression level of CYP1A1 and CYP1A2 mRNA.

This observed regulation pattern toward EROD is very different to that obtained with the direct incubation of rat liver microsomes with ITCs. Conaway et al. (1996) found that the aromatic BITC and PEITC had a greater inhibitory affect on EROD than that produced by the aliphatic AITC and SFN. However, in experiments with rat and human hepatocytes SFN reduced CYP1A1/1A2-dependent EROD activity, whereas the aromatic PEITC induced the expression of the CYP1A1 and 1A2 genes, in agreement with our results (Mahéo et al., 1997; Gross-Steinmeyer et al., 2010).

The observed induction of EROD activity and CYP 1A1 and 1A2 transcripts by the aromatic BITC, HBITC and PEITC was likely mediated by AhR, a transcriptional factor known to be activated by aromatic planar ligands and in particular by polycyclic aromatic hydrocarbons (Denison and Nagy, 2003). In hepatocyte cultures, the transcriptional increase of CYP1A1 and 1A2 genes may overwhelm the original mechanism-based inhibition by the aromatic ITCs of corresponding microsomal enzymes.

On the other hand, the observed inhibition of EROD by SFN and IBITC, may be explained by the fact that both compounds are rather hydrophilic [log p = 0.15–0.17 (Vermeulen et al., 2009)] compared to the other ITCs used [log p = 1.9–3.2] and might have produced higher cellular accumulation levels as suggested by a study on the SFN, AITC, BITC and PEITC in HepG2/EpRE-TR-GFP cells (Ye and Zhang, 2001). It should be noted that a portion of ITCs are present in the cells as thiol conjugates which have their own formation and deconjugation kinetics and have less inhibition power than that of the parent ITCs (Conaway et al., 2002).

Unexpectedly, our results revealed a strong inhibition of microsomal BQD activity, a marker of CYP3As, in response to all the ITC solutions without any clear relation to chemical structure, contrasting therefore with results obtained by Hanlon et al. (2008) in rat liver slices, where SFN treatment up to 50 μM failed to affect
the BQD activity. Our semi-quantitative RT-PCR analysis showed that the inhibition of BQD was likely due to a marked decrease of CYP3A2 gene expression (CYP3A2 is the major CYP gene belonging to the CYP3A subfamily in rat liver) after 4 h ITC treatment. The inhibition of a CYP3A (CYP3A4) at the transcriptional level has also been described in human hepatocytes treated with SFN (Mahéo et al., 1997; Gross-Steinmeyer et al., 2010). It is interesting to note that suppression of BQD activity by ITCs might not be occurring at the protein level through a mechanism-based inhibition considering that there is evidence that in rat liver microsomes BITC does not inhibit the CYP3A2 activity (Goosen et al., 2001). Since SFN has been reported to be an effective antagonist of human PXR (Zhou et al., 2007), an hepatic transcriptional factor that regulates the expression of CYP3A genes, our observed reduction in CYP3A2 mRNA might be due to a direct effect of ITCs on rat PXR.

Taking into account that CYP3A4 is involved in the biotransformation of over 50% of all therapeutic drugs, the inhibition of CY-P3As by ITCs could have an important influence on drug–drug interactions, bioactivation of CYP3A-dependent carcinogens and other PXR-regulated biotransformation pathways. For example treatment with SFN and PEITC in human hepatocytes was found to reduce the bioactivation of the hepatocarcinogenic mycotoxin aflatoxin B1 to the genotoxic form: aflatoxin B1-8,9-oxide, a reaction catalyzed by CYP3A4 (Gross-Steinmeyer et al., 2010).

However, the chemopreventive effects of ITCs may not necessarily involve modulation of CYP-mediated bioactivation of carcinogens. A major protective mechanism of ITCs is the induction of antioxidant and phase II ARE-driven genes such as NQO1, HO-1 and GST, which are under control of the transcription factor Nrf2. Most studies screening the cancer chemopreventing properties of electrophilic compounds including ITCs are based on induction of NQO1 and other genes in various hepatoma cell lines (Postner et al., 1994; Ahn et al., 2010).

In general the induction potency of these compounds towards phase II enzymes was related to their chemical reactivity with the sulfhydryl groups (Dinkova-Kostova et al., 2002). However, the degree of induction of these enzymes by ITCs was found to vary with the cell lines and was thought to be dependent on their overall intracellular accumulation levels (Ye and Zhang, 2001).

Our results in primary rat hepatocytes, which are a better model than hepatoma cell lines for the intact liver may help shed light on the structure–activity relationship of ITCs on Nrf2/ARE-dependent gene expression.

In agreement with earlier studies in rat hepatocytes (Mahéo et al., 1997; Payen et al., 2001), we found that SFN increases the activity not only of NQO1 and GST but also of other antioxidant/detoxifying enzymes such as HO-1, GSSG-red and catalase. As was previously observed in hepatoma cell lines (Jeong et al., 2005), we have shown that in the rat hepatocytes the induction of these enzymes also occurred through the activation of Nrf2 protein and its subsequent translocation into the nucleus (Fig. 7). Our findings show that all the ITCs tested increased the HO-1 and NQO1 enzymes at transcriptional and activity levels. On the other hand, the activities of GST, GSSG-red and catalase were significantly increased only by some ITCs. Indeed, AITC, BITC and HBITC failed to induce the GST activity, whereas BITC and HBITC did not induce GSSG-red activity, and AITC failed to induce catalase.

The ability of ITCs to induce NQO1 and other enzymes did not appear to be linked to hydrophilicity or presence of an aromatic ring, whereas the ability of ITCs to modulate CYPs did seem to depend on ITC structure. The disparity in the enzymatic induction by ITCs is probably due to their different cellular uptake, metabolism and intermediates formation (e.g. glutathione conjugates). The bio-transformation process is an important issue for understanding the efficiency, mode of action and potential toxicity of ITCs as chemopreventive agents (Conaway et al., 2002).

In the current study, we demonstrated with immunoblot that all the 40 μM ITCs were able to activate Nrf2 after just 1 h of treatment, though to different degrees, and thus leading to its partial translocation into the nucleus. Of course the differences observed in Nrf2 activation in the rat hepatocytes, could be due to a different time dependence with a consequent impact on the expression of Nrf2-target genes as found in hepatoma HepG2 cells (Jeong et al., 2005). However, it should be noted that other mechanisms not mentioned here might be involved in the regulation of the antioxidant and phase II genes, especially in the liver, in addition to the ARE/Nrf2-mediated pathway.

In conclusion, this study has established for the first time in primary rat hepatocytes that ITCs with various chemical structures are able to modulate, although in different ways, CYPs, antioxidant and phase II enzymes and activate Nrf2. This data supports the suggestion put forward by Keum et al. (2008) that ITCs, as a class of electrophilic compounds, are general activators of ARE/Nrf2 regulated genes.

Conflict of Interest

The authors declare that there are no conflicts of interest.

References


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