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# Reactive Oxygen Species-Related Induction of Multidrug Resistance-Associated Protein 2 Expression in Primary Hepatocytes Exposed to Sulforaphane

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**Expression of multidrug resistance-associated protein 2 (MRP2), an efflux pump contributing to biliary secretion of xenobiotics, was investigated in primary rat and human hepatocytes exposed to sulforaphane, a naturally-occurring chemopreventive agent. Northern blot indicated that sulforaphane increased MRP2 mRNA levels in primary rat hepatocytes; it also induced expression of drug metabolizing enzymes such as glutathione S-transferase A1/2 isoforms and NAD(P)H:quinone oxidoreductase in a dose-response and time-course manner similar to that observed for the upregulation of MRP2 transcripts. This sulforaphane-related increase of MRP2 mRNAs paralleled increased expression of 190 kD MRP2 protein as assessed by Western blotting; it was fully abolished by the transcription inhibitor actinomycin D. MRP2 induction was associated with increased cellular production of reactive oxygen species (ROS) and addition of dimethyl sulfoxide, that reduced sulforaphane-related formation of ROS, and also decreased MRP2 mRNA levels in sulforaphane-treated primary rat hepatocytes; this suggests that sulforaphane-mediated production of ROS may contribute to MRP2 induction. This link between ROS and MRP2 regulation was further supported by the increase of MRP2 expression occurring in response to t-butylhydroquinone, known to regulate drug metabolizing enzymes through ROS formation. In addition to rat cells, primary human hepatocytes exposed to sulforaphane also displayed induced MRP2 expression evidenced at both mRNA and protein levels. All these observations strongly support the conclusion that the export pump MRP2 can be classified among the detoxifying proteins that are regulated by sulforaphane and that are thought to contribute, at least in part, to its anticarcinogenic properties.** 

***Key Words:*** **chemopreventive agent; human cells; multidrug resistance-associated protein 2; primary hepatocytes; reactive oxygen species (ROS); sulforaphane.**

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Multidrug resistance-associated protein 2 (MRP2), previously called canalicular multispecific organic anion transporter (cMOAT), is a 190 kDa export pump predominantly found at the biliary pole of hepatocytes (1, 2). This ATP-binding cassette membrane protein belongs to the MRP transporter subfamily comprizing other efflux pumps such as MRP1 and MRP3 (3). MRP2 mediates the passage from hepatocytes into the bile of various types of organic anions, including glucuronate, sulphate and glutathione conjugates (4, 5). Mutant rat strains lacking MRP2 expression such as the transport deficient (TR-) Wistar rat and the Eisai hyperbilirubinemic rat (EHBR) therefore display low biliary secretion of conjugates of xenobiotics and endogenous compounds such as bilirubin (1, 6). Similarly, a hereditary deficiency in human MRP2 expression, known as the Dubin-Johnson syndrome, causes reduced biliary elimination of bilirubin glucuronides (7).

Regulation of hepatic expression of MRP2 is still poorly understood. Recent studies have however indicated that some inducers of liver drug metabolizing enzymes increase MRP2 levels. Indeed, the potent glucocorticoid dexamethasone has been shown to upregulate expression of both cytochrome P450 3A1/2 and MRP2 in rat liver (8, 9). In the same way, oltipraz, a synthetic 1,2-dithiole-3-thione displaying chemopreventive properties towards chemical carcinogenesis and known to induce hepatic levels of drug metabolizing enzymes such as NAD(P)H:quinone oxidoreductase (QR) and glutathione S-transferase (GST) isoforms including GSTA1/2 (10, 11), has been demonstrated to increase MRP2 amounts in primary rat hepatocytes (12). Whether such MRP2 upregulation is restricted to oltipraz among chemopreventive agents or, on the contrary, also occurs in response to other compounds exhibiting anticarcinogenic activities, in particular natural agents found in the food, is unknown; responsiveness of human hepatocytes and putative involvement of reactive oxygen species (ROS), that play a major role in chemopreventive agent-mediated induc-



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tion of drug metabolizing enzymes (13), also remain to be clarified. To address these questions, we have investigated in the present study MRP2 expression in primary rat and human hepatocytes exposed to sulforaphane. This compound was retained since (i) it belongs to a class of chemopreventive agents, i.e., isothiocyanates, distinct from that of oltipraz (14), (ii) it occurs naturally in widely consumed vegetables, especially broccoli (15), (iii) its blocking effects towards chemical-initiated tumor formation have been well demonstrated (16), (iv) it is a very potent inducer of phase II conjugating enzymes in both human and rodent hepatocytes, thereby most likely increasing hepatic formation of MRP2 substrates such as drug conjugates (17), and (v) it leads to increased production of cellular ROS in various cell types (18). Our data demonstrate that sulforaphane markedly increases MRP2 expression in human and rat primary hepatocytes through a mechanism that likely involves, at least in part, ROS production.

## MATERIALS AND METHODS

*Chemicals.* t-Butylhydroquinone (tBHQ), actinomycin D, and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). Sulforaphane was obtained from LKT Laboratories (St Paul, MN).

*Cell isolation and culture.* Hepatocytes from male Sprague Dawley rats weighing 150–200 g were isolated by liver perfusion as previously described except that a liberase solution was used for dissociation of liver cells instead of a collagenase solution (19). Human hepatocytes from five adult donors undergoing resection for primary and secondary tumors were obtained by perfusion using a collagenase solution (20). All experimental procedures complied with French laws and regulations and were approved by the National Ethics Committee. Cells were seeded at a density of $10^5$ cells/cm$^2$ in plastic dishes in Williams'E medium supplemented with 0.2 mg/ml bovine serum albumin, 10 mg/ml bovine insulin, and 10% (v/v) fetal calf serum. The medium was discarded 4 h after rat hepatocyte seeding and 24 h after human cell seeding and cells were thereafter maintained in serum-free medium supplemented with $10^{-7}$ M dexamethasone.

*Isolation of total RNAs and Northern blot analysis.* Total RNAs were extracted from cultured hepatocytes as previously described (21). Ten $\mu$g of total RNAs were used for electrophoresis in a denaturing 6% (v/v) formaldehyde-1.2% (w/v) agarose gel and were thereafter transferred onto Hybond-N+ nylon filters (Amersham, Arlington, Heights, IL). After prehybridization, the filters were hybridized with $^{32}$P-labelled cDNA probes, washed, dried, and autoradiographed at −80°C. Specific cDNA probes used for the analysis of GSTA1/2 and MRP2 mRNAs were prepared by reverse transcription-polymerase chain reaction as previously reported (22, 23) whereas QR mRNAs were detected using the pDTD55 probe (24). Equal gel loading and efficiency of the transfer were checked using an 18S rRNA probe.

*Preparation of crude membranes and western blotting.* Crude membranes were prepared from cultured hepatocytes by differential centrifugation as described by Germann *et al.* (25). Membrane proteins were then separated on a 7.5% polyacrylamide gel and electrophoretically transferred to nitrocellulose sheets. Sheets were blocked 2 h with Tris-buffered saline containing 3% bovine serum albumin and 10% milk and were next incubated with the rabbit anti-rat MRP2 polyclonal antibody RM2 (8) or with the mouse anti-human MRP2 monoclonal antibody $M_2$III-6 (1) kindly provided by Professor R. Scheper (Academish Ziekenhuis Vrije Universiteit, Amsterdam, The Netherlands). A peroxidase-conjugated anti-rabbit or anti-mouse antibody was thereafter used as secondary antibody. After washing, blots were developed by chemiluminescence using the Amersham ECL detection system. Control blot was performed in parallel using the same protocol, except that the anti-MRP2 antibody was replaced by a nonimmune serum.

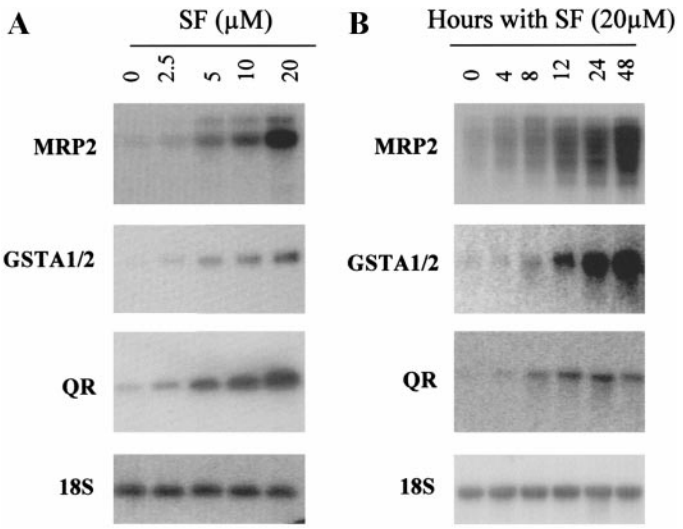


**FIG. 1.** Dose dependence (A) and time-course (B) of MRP2 mRNA induction in response to sulforaphane treatment in primary rat hepatocytes. Each well contains 10 $\mu$g total RNA isolated from primary cultured rat hepatocytes exposed to various doses of sulforaphane (SF) (0 to 20 $\mu$M) for 48 h (A) or to 20 $\mu$M SF for various lengths of time (0 to 48 h) (B). RNAs were then transferred to Hybond N+ sheets and hybridized with MRP2, GSTA1/2, QR, and 18S probes. The results shown are representative of two independent experiments.

*Determination of ROS formation.* Analysis of ROS production was performed using the dihydrorhodamine 123 (DHR) assay (26, 27). Briefly, cells plated in 96-well microplates were incubated with 5 $\mu$M DHR for 24 h. DHR, an uncharged and nonfluorescent compound, passively diffuses into cells and is subsequently oxidized upon the action of cellular ROS into rhodamine 123 (Rh 123), a cationic fluorescent dye. Cellular Rh 123-related fluorescence, which reflects ROS formation, was thereafter measured using a Spectra Max Gemini spectrofluorimeter (Molecular Devices Sunnyvale, CA); excitation and emission wavelengths were 488 nm and 538 nm, respectively.

*Statistical analysis.* ROS-related fluorescence data were analyzed by the Student's *t*-test. The criterion of significance between the means was $P < 0.05$.

## RESULTS

Primary rat hepatocytes were first treated by various concentrations of sulforaphane ranging from 2.5 to 20 $\mu$M for 48 h. Northern blot analysis indicated that the chemopreventive agent strongly enhanced MRP2 mRNA levels in a dose-dependent manner (Fig. 1A); indeed, this effect began with 2.5 $\mu$M sulforaphane and was maximal when the chemical was used at 20 $\mu$M. As previously reported (8), several MRP2 mRNAs of dif-

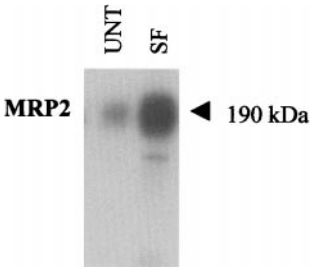


**FIG. 2.** Western blot analysis of crude membrane proteins obtained from primary rat hepatocytes exposed to sulforaphane. Crude membrane fractions were prepared from primary rat hepatocytes either untreated (UNT) or exposed to 20 $\mu$M sulforaphane (SF) for 48 h. Membrane proteins were then separated on 7.5% polyacrylamide gels and transferred onto nitrocellulose sheets. Expression of MRP2 was further investigated by immunoblotting as described under Materials and Methods. The results shown are representative of four independent experiments.

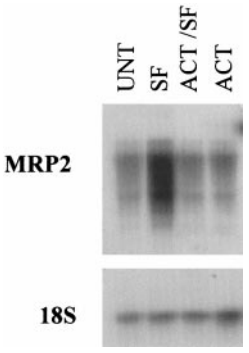


**FIG. 3.** Effect of actinomycin D on sulforaphane-mediated MRP2 mRNA induction. Each well contains 10 $\mu$g total RNAs isolated from primary rat hepatocytes either untreated (UNT) or exposed to 20 $\mu$M sulforaphane (SF) or 5 $\mu$g/ml actinomycin D (ACT) or cotreated with SF and ACT for 10 h. RNAs were transferred to Hybond N+ sheets after electrophoresis and hybridized with MRP2 and 18S probes. The results shown are representative of two independent experiments.

ferent sizes, especially of 5.5 kb and 7.5 kb, were evidenced on the blot; they likely corresponded to alternative mRNA splicing variants with different 3′-untranslated region lenghts (28) and their relative proportions were not modified in response to sulforaphane whatever the concentrations used. In addition to MRP2 mRNA levels, sulforaphane was found to increase amounts of mRNAs of drug metabolizing enzymes such as GSTAl/2 and QR already known to be regulated by chemopreventive agents (15, 17); dose-responses corresponding to the induction of these detoxifying enzyme transcripts were similar to that occurring for MRP2 mRNA upregulation (Fig. 1A). Time-course of the induction of MRP2 mRNA levels in rat hepatocytes treated by 20 $\mu$M sulforaphane was thereafter studied. Northern blot analysis indicated that MRP2 mRNA amounts were up-regulated after a 4 h exposure to the chemopreventive agent (Fig. 1B). Longer exposures (12 to 48 h) were however required to obtain maximal induction of MRP2 mRNA levels (Fig. 1B). Such lenghts of exposure were also needed for maximal up-regulation of GSTA1/2 and QR (Fig. 1B).

Crude membrane fractions were further prepared from primary rat hepatocytes exposed to 20 $\mu$M sulforaphane for 48 h and were used to investigate MRP2 expression by Western blot analysis (Fig. 2). A RM2-antibody-reactive band of 190 kDa corresponding to MRP2 was markedly overexpressed in sulforaphane-treated hepatocytes when compared to their untreated counterparts whereas this band was not present in control blots performed using non-immune rabbit serum as a primary antibody (data not shown).

The effect of actinomycin D, a known inhibitor of transcription, on sulforaphane-mediated induction of MRP2 mRNA levels was thereafter evaluated by Northern blotting (Fig. 3). Actinomycin D, used at a concentration (5 $\mu$g/ml) likely decreasing RNA synthesis levels to less than 1% of values found in untreated liver cells (29), fully suppressed the MRP2 mRNA increase occurring in sulforaphane-treated hepatocytes whereas it did not affect basal amounts of MRP2 transcripts.

Since regulation of GSTA1/2 and QR expression in response to chemopreventive agents including isothiocyanates has been shown to involve ROS formation (13), we next determined ROS production in primary rat hepatocytes exposed to sulforaphane for 24 h. As shown in Fig. 4, sulforaphane strongly increased the cellular generation of ROS in primary rat hepatocytes. Addition of 2% DMSO, a known radical scavenger (30), however diminished cellular ROS amounts in SF-treated hepatocytes; it also reduced basal production of ROS in hepatocytes not exposed to sulforaphane. We further examined the effect of DMSO on sulforaphane-

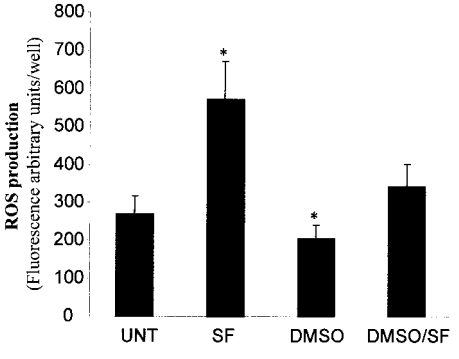


**FIG. 4.** ROS production in sulforaphane-treated rat hepatocytes. Primary rat hepatocytes plated in 96-well microplates were either untreated (UNT) or treated by either 20 $\mu$M sulforaphane (SF), 2 % (v/v) DMSO, or both SF and DMSO for 24 h. Cellular ROS formation was then determined using the DHR assay as described under Materials and Methods. ROS production was expressed as fluorescence arbitrary units/well; data are mean ± SD of three independent experiments in triplicate. * $P < 0.05$ when compared to untreated hepatocytes.

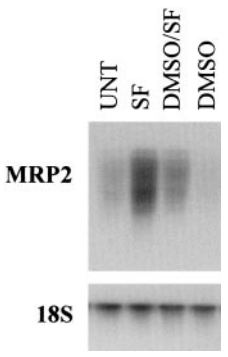


**FIG. 5.** Effect of DMSO on sulforaphane-mediated MRP2 mRNA induction. Each well contains 10 $\mu$g total RNAs isolated from primary rat hepatocytes either untreated (UNT) or exposed to 20 $\mu$M sulforaphane (SF) or 2% (v/v) DMSO or cotreated with SF and DMSO for 48 h. RNAs were transferred to Hybond N+ sheets after electrophoresis and hybridized with MRP2 and 18S probes. The results shown are representative of three independent experiments.

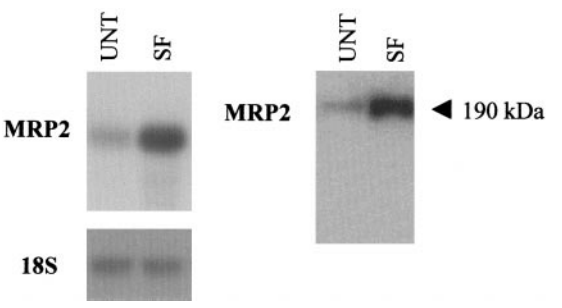


**FIG. 7.** Induction of MRP2 gene expression in sulforaphane-treated primary human hepatocytes. Primary human hepatocytes were either untreated (UNT) or exposed to 50 $\mu$M sulforaphane (SF) for 72 h. MRP2 mRNA levels (A) and MRP2 amounts (B) were then analyzed by Northern and Western blotting, respectively, as described under Materials and Methods. The results shown are from one human hepatocyte population; similar data were also obtained with hepatocytes from two additional donors.

related MRP2 gene regulation. As indicated in Fig. 5, DMSO was found to decrease MRP2 mRNA levels in SF-treated hepatocytes; it also slightly downregulated basal expression of MRP2 transcripts. We next analyzed the effects of t-BHQ, a compound well-known to induce GSTA1/2 gene expression through ROS production (13), on MRP2 expression in rat hepatocytes. This compound used at 100 $\mu$M for 48 h enhanced MRP2 mRNA levels as demonstrated by Northern blot analysis (Fig. 6A); in parallel, it also augmented GSTA1/2 and QR gene expression (Fig. 6A) and Western blotting evidenced increased MRP2 amounts in t-BHQ-treated rat hepatocytes when compared to their untreated counterparts (Fig. 6B).

We finally investigated using primary cultures of human hepatocytes whether sulforaphane-mediated MRP2 upregulation reported above in rat cells may also occur in human cells. Northern and Western blot analyses indicated increased expression of MRP2 gene in sulforaphane-treated primary human hepatocytes when compared to their untreated counterparts (Fig. 7).

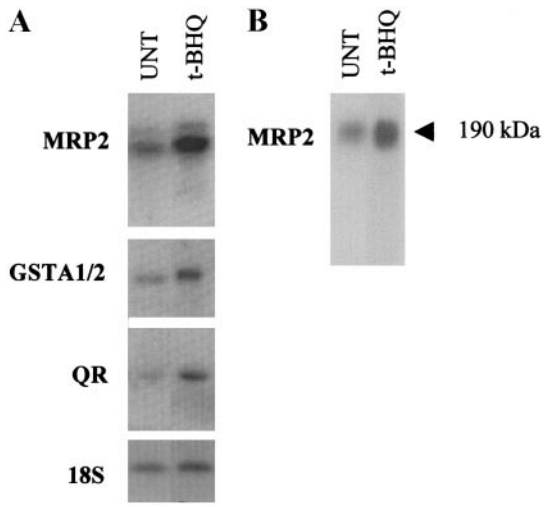


**FIG. 6.** Effect of t-BHQ on MRP2 gene expression in primary rat hepatocytes. Primary rat hepatocytes were either untreated (UNT) or exposed to 100 $\mu$M t-BHQ for 48 h. MRP2, GSTA1/2, and QR mRNA levels were thereafter analyzed by Northern blotting (A) whereas MRP2 amounts were investigated by Western blotting (B) as reported under Materials and Methods. The data shown are representative of three independent experiments.

## DISCUSSION

The results reported in the present study demonstrate for the first time according to the best of our knowledge that the naturally-occurring chemopreventive agent sulforaphane is a powerful inducer of the export pump MRP2 in liver parenchymal cells, including human ones. Indeed exposure of both primary rat and human hepatocytes to this isothiocyanate found at rather elevated concentrations in some vegetables such as crucifers resulted in a markedly-increased expression of MRP2 at both mRNA and protein levels. We recently reported that oltipraz, a synthetic 1,2-dithiole-3-thione originally developed as an anti-schistosomal agent and well known for its chemopreventive properties, similarly upregulated MRP2 levels in rat hepatocytes (12). Since sulforaphane and oltipraz are structurally unrelated, these data indicate that chemopreventive agents belonging to different chemical classes and already known for altering expression of several hepatic detoxifying enzymes such as GST and UDP-glucuronosyl transferase isoforms, QR and some cytochromes P450 (10, 11, 13, 17, 31), likely increase in parallel expression of the export pump MRP2.

Sulforaphane action on MRP2 mRNA levels requires active gene transcription since it was fully blocked by actinomycin D. Similarly, the effects of chemopreven-

tive agents on drug metabolizing enzymes such as GSTA1/2 have been shown to involve transcriptional events, including increased GSTA1/2 gene transcription as recently demonstrated by run-on experiments (22).

Previous studies have suggested that chemopreventive agents could upregulate expression of detoxifying enzymes through production of ROS (13, 32). Indeed, these compounds induce oxidative stress and the resulting imbalanced cellular redox state appears to be the principal signal that leads to the transcriptional activation of genes encoding drug metabolizing enzymes (13, 33). Such a regulation involves cis-acting regulatory elements found in the promoter of the responsive genes and termed antioxidant response elements (ARE) (34). Interestingly, the features of MRP2 mRNA upregulation by sulforaphane in primary rat hepatocytes, i.e., dose-response and time-course of the induction, were demonstrated to be similar to those found for sulforaphane-related induction of GSTA1/2 and QR transcripts. These data suggest that the cellular and molecular mechanisms by which sulforaphane acts on MRP2 expression may be, at least in part, closed to the ROS-dependent processes involved in regulation of GSTA1/2 and QR by chemopreventive agents. Several other arguments support this hypothesis: (i) sulforaphane efficiently enhanced ROS formation in primary hepatocytes when used at a concentration inducing MRP2 expression, (ii) the radical scavenger DMSO decreased levels of both ROS and MRP2 mRNAs in sulforaphane-treated rat hepatocytes and we have observed that treatment by the antioxidant *N*-acetylcysteine also inhibited MRP2 induction due to sulforaphane (data not shown), (iii) DMSO also diminished basal levels of both cellular ROS and MRP2 mRNAs in primary hepatocytes, indicating that MRP2 expression may be at least in part controlled by the cellular redox status in such cells, (iv) t-BHQ, well-known to induce drug metabolizing enzymes such as GSTA1/2 and QR through production of ROS (13, 35), increased MRP2 expression in rat hepatocytes, and (v) an ARE-like sequence has been recently described in the 5′-flanking region of the human MRP2 gene (36). In addition, it is noteworthy that the export pump MRP1, whose functional features, including the substrates handled, are closed from those of MRP2, has already been shown to be upregulated by oxidant agents (27). It should however be kept in mind that chemopreventive agents can also alter hepatic detoxifying proteins, especially some cytochromes P450, through mechanisms that are thought to be independent of ARE- and ROS-related processes (37). Further studies are therefore needed to more precisely clarify the mechanisms by which oxidant agents, including chemopreventive compounds, can induce expression of some members of the MRP subfamily.

The putative contribution of MRP2 upregulation to the chemopreventive properties of sulforaphane remains to be determined. It can however be hypothesized that sulforaphane-mediated coinduction of drug metabolizing enzymes and MRP2 is a coordinate cellular response leading firstly to increased metabolic inactivation of chemical carcinogens through formation of conjugates and secondly to enhanced MRP2-related biliary secretion of these conjugates. *In vivo* experiments, including analysis of biliary elimination of chemical carcinogen conjugates, are however required to validate such an hypothesis. Nevertheless, it is noteworthy that sulforaphane, that is thought to be at least as effective as oltipraz in chemoprotection against carcinogenesis due to chemicals such as aflatoxin $B_1$ in rodents (16, 38), induced expression of MRP2 in primary human hepatocytes; it also increased levels of phase II metabolizing enzymes including GST isoforms in such cells as in rat hepatocytes (17). These data are therefore consistent with efficient upregulation of human detoxifying proteins in response to sulforaphane and reinforced the idea that this chemopreventive agent widely distributed in consumed vegetables may exhibit potential anticarcinogenic effects in humans.

In summary, our results demonstrate that treatment by the chemopreventive agent sulforaphane increased expression of the conjugate export pump MRP2 in rat and human primary hepatocytes. Such a regulation seems to involve, at least in part, increased ROS production in rat hepatocytes and it may contribute to the anticarcinogenic properties of sulforaphane.

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