Modulation of Glucose-6-phosphate Dehydrogenase Activity and Expression Is Associated with Aryl Hydrocarbon Resistance in Vitro*

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The mutagenic effect of environmental carcinogens has been well documented in animal models and in human studies but the mechanisms involved in preventing carcinogen insult have not been fully elucidated. In this study we examined the molecular and biochemical changes associated with carcinogen resistance in a series of aryl hydrocarbon-resistant MCF-7 cell lines developed by exposure to benz[a]pyrene (BP). The cell lines were designated as AH1, AH2, and AH3 to denote their increasing fold resistance to BP compared with wild type cells. These cell lines were also resistant to another aryl hydrocarbon (AH), dimethyl benz[a]anthracene, but not to pleiotropic drugs (doxorubicin, vinblastine, and taxol). The resistant cell lines showed an increase in the level of the primary intracellular antioxidant, reduced glutathione, corresponding to increasing AH resistance. However, there was no change in glutathione reductase activity. The generation of reduced glutathione requires NADPH, and we therefore examined the activity and expression of the rate-limiting enzyme in NADPH production, glucose-6-phosphate dehydrogenase (G6PD). An increase in G6PD specific activity was associated with increasing aryl hydrocarbon resistance. This was due to an increased expression of G6PD in resistant cells, which was demonstrated by increases in both protein and mRNA levels. However, there was no increase in the transcription rate of G6PD in the resistant cell lines, indicating that the increase G6PD expression is due to a post-transcriptional modulation, which was confirmed by actinomycin D chase experiments. These results demonstrate that modulation of G6PD expression and activity is an important mechanism in AH resistance.

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1 The abbreviations used are: BP, benzo[a]pyrene; AH, aryl hydrocarbons; AH*, aryl hydrocarbon resistant cells; DMBA, dimethylbenz[a]anthracene; G6PD, glucose-6-phosphate dehydrogenase; GSH, glutathione; PPP, pentose phosphate pathway; NADPH, nicotinamide adenine dinucleotide, reduced form; GST, glutathione S-transferase; QR, quinone reductase; RT-PCR, reverse transcriptase-polymerase chain reaction.

mental carcinogen that humans are exposed to in cigarette smoke, industrial by-products, and cooked meat (1). It is regarded as a human carcinogen, since BP-DNA adducts have been detected in humans (2), and BP metabolites have been shown to bind to specific DNA residues in the p53 gene known as “hot spots” of mutation in human cancers (3). BP is metabolized to genotoxic derivatives by the action of Phase 1 enzymes, and these metabolites are detoxified by the Phase 2 enzymes, such as quinone reductase (4), UDP-glucuronosyltransferase (5), and glutathione S-transferase (6). Both Phase 1 and Phase 2 enzymes require NADPH as a co-factor. NADPH is the primary intracellular reductant whose synthesis is regulated by the oxidative arm of glucose metabolism, the pentose phosphate pathway (PPP). The rate-limiting enzyme of the PPP (Fig. 1) is glucose-6-phosphate dehydrogenase (G6PD) (7), whose major physiological role is to supply NADPH by the conversion of glucose 6-phosphate (Glu-6-P) to 6-phosphogluconate. NADPH is also generated by the next step in the PPP, the conversion of 6-phosphogluconate to ribulose 5-phosphate by 6-phosphogluconate dehydrogenase. The essentiality of G6PD in mammalian cells has been well recognized. It is highly conserved during evolution (8), and it has been shown to be necessary for cell growth (9). G6PD, normally thought of as a “housekeeping” enzyme whose expression is constitutive, is, in fact, induced by agents which cause oxidative stress (10, 11) and is essential for resistance to oxidative stress (12). G6PD is also induced by other agents such as insulin and epidermal growth factor (13) and by dietary conditions (14, 15).

Because of the essential role of NADPH in carcinogen detoxification, we hypothesize that modulation of G6PD activity may be directly associated with carcinogen resistance. To test this hypothesis, we developed a series of aryl hydrocarbon-resistant (AH*) cells derived from MCF-7 human breast epithelial cancer cells by continuous exposure to BP. These cells were grown in escalating concentrations of BP, reserving surviving cells at each level of BP. We thereby generated a series of cells with increasing resistance to the cytotoxic effects of BP. These cell lines were also resistant to another AH, dimethylbenz[a]anthracene (DMBA) but not to pleiotropic drugs (doxorubicin, vinblastine, and taxol). We found that AH resistance is associated with changes in G6PD activity and expression, indicating that G6PD is a central mechanism involved in carcinogen resistance.

EXPERIMENTAL PROCEDURES

Materials—Dicoumarol, Glu-6-P, NADP+, NADPH, G6PD, anti-G6PD, Tween 20, flavin adenine dinucleotide, glutathione reductase, oxidized glutathione, 2,6-dichloroindophenol, BP, DMBA, menadione, and dimethyl sulfoxide were purchased from Sigma. Colorimetric assay for glutathione reductase was from Oxis International, Inc. (Portland, OR).
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Fig. 1. The PPP. The PPP is shown coupled with the glutathione cycle and quinone reductase detoxification reaction. GR, glutathione reductase.

Fig. 2. IC₅₀ for BP and DMBA. The concentration of BP or DMBA at which cell growth was inhibited by 50% (IC₅₀) was determined by growth inhibition assays as described under “Experimental Procedures.” Cells were counted daily for 5 continuous days, and the values are given as the mean ± S.E. of three determinations.

OR and glutathione assay kit was from Calbiochem (San Diego, CA). Omniscript reverse transcription-polymerase chain reaction (RT-PCR) kit was from Qiagen (Valencia, CA). Primers for G6PD RT-PCR were from Stratagene (La Jolla, CA). RPMI 1640, glutamine, fetal bovine serum, trypsin/EDTA, normal saline, TBE buffer, Trizol, and LipofectAMINE were from Life Technologies, Inc. (Gaithersburg, MD). The β-galactosidase-containing reporter vector was from CLONTECH (Palo Alto, CA). 10% TBE gels, sample and running buffers were from Novex (San Diego, CA).

Cell Culture—To generate MCF-7 cell lines with different levels of resistance to AH, wild type (WT) MCF-7 cells were initially grown and serially passed in 0.05 M BP. A stock of the surviving cells was preserved by freezing in RPMI 1640 supplemented with 20% fetal bovine serum, 2 mM glutamine, and 10% dimethyl sulfoxide. The remaining cells were then grown with stepwise increases of BP concentrations for 6–9 months, with a stock of surviving cells preserved by freezing after each increase in BP. From the many resistant subclones thus generated, we selected 3 for this study, demonstrating 40-, 100-, and 200-fold increase in the BP concentration needed to inhibit cell growth by 50% (IC₅₀). These cell lines were designated AH(R₄₀), AH(R₁₀₀), and AH(R₂₀₀). The AH(R) cell lines were maintained by continuous exposure to 0.05 M BP. A stock of the surviving cells was preserved by freezing in RPMI 1640 supplemented with 20% fetal bovine serum, 2 mM glutamine, and 10% dimethyl sulfoxide. The remaining cells were then grown with stepwise increases of BP concentrations for 6–9 months, with a stock of surviving cells preserved by freezing after each increase in BP. From the many resistant subclones thus generated, we selected 3 for this study, demonstrating 40-, 100-, and 200-fold increase in the BP concentration needed to inhibit cell growth by 50% (IC₅₀). These cell lines were designated AH(R₄₀), AH(R₁₀₀), and AH(R₂₀₀). The AH(R) cell lines were maintained by continuous exposure to their IC₅₀ concentration of BP for five passages, and the cells were subsequently passed in BP-free medium for at least 4 passages prior to experimental use. For the experiments, AH(R) cells in the 4th to the 10th passages without BP were used. All experiments were carried out on 75% confluent cells in RPMI 1640 containing 10% fetal bovine serum and 2 mM glutamine, unless otherwise indicated.

Growth Inhibition Studies—Cells were plated in triplicate at 500,000 cell/25-cm² flask in RPMI 1640 with 10% fetal calf serum and 2 mM glutamine. BP or DMBA dissolved in dimethyl sulfoxide was added at different concentrations after 24 h of plating. Cells were counted by hemocytometer daily for 5 continuous days.

Enzyme Assays—Enzyme assays were performed using the cytosolic fraction of each cell line. Cytosol was prepared as follows: the cells were washed twice with normal saline (0.9% NaCl), scraped, and pelleted by centrifugation at 500 × g for 10 min at 4 °C. The pellet was resuspended in normal saline and sonicated for 45 s on ice using a Branson sonifier at setting 2. The sonicate was then centrifuged at 500,000 × g for 15 min at 4 °C and the supernatant saved and stored at −80 °C until use. The protein was assayed by the method of Bradford (16) and enzyme assays were conducted within 1 week of the sample preparation. G6PD activity was assayed by spectrophotometric measurement of the rate of appearance of NADPH at 340 nm (17). Glutathione reductase (18), glutathione peroxidase (19), catalase (20), quinone reductase (QR) (21), and glutathione S-transferase (GST) (22) activities were measured as described.

Glutathione Measurement—Reduced glutathione (GSH) was measured colorimetrically using a kit from Calbiochem as directed.

G6PD Determination by Western Immunoblotting—Cell cytosolic protein (15 μg) from WT and AH(R) cells was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membrane 0.2 μm (Trans-Blot Transfer Medium, Bio-Rad, Hercules, CA). The membrane was blocked with 5% nonfat dry milk, exposed to an anti-G6PD antibody, treated with goat anti-rabbit conjugated to horseradish peroxidase, and then developed using ECL chemiluminescence kit as directed (Amersham, Piscataway, NJ). Quantitation of the film image was achieved by scanning densitometry.

Northern Blot Analysis—Total RNA was isolated from WT and AH(R) cells using guanidine isothiocyanate and a cesium chloride gradient as reported previously (23). Total RNA (15 μg) was applied to a denaturing 2.2% formaldehyde, 1% agarose gel. After transfer onto nylon membrane, the membrane was hybridized at 42 °C overnight with [³²P]dCTP-labeled G6PD cDNA probes (24). Probes were labeled by random priming (25).

G6PD-promoter Controlled Transcription—A 1.2-kilobase fragment of the human G6PD proximal promoter was amplified with primers chosen using OligoPrimer Analysis (National Biosciences Inc., Plym-
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**Phase II enzyme activities in human breast cancer MCF-7 wild type and AH-resistant (AH-R) cells**

| Cells | GST c.u./mg | GST-\( \tau \) ng/ml | Glutathione peroxidase k/mg | Catalase k/mg | QR nm/min/mg | Glutathione reductase nm/min/mg | 6-phosphogluconate dehydrogenase nm/min/mg |
|-------|-------------|-------------------|---------------------|--------------|--------------|-------------------------------|------------------------------------------|
| WT    | 3.2 ± 0.12  | <0.78             | 2.1 ± 0.32          | 0.2 ± 0.06   | 136 ± 2.8    | 8.2 ± 1.3                     | 31.3 ± 1.02                              |
| AH\( ^{40} \) | 3.7 ± 0.13  | <0.78             | 4.6 ± 0.44          | 0.3 ± 0.02   | 128 ± 1.3    | 6.9 ± 0.97                    | 27.2 ± 2.04                              |
| AH\( ^{100} \) | 2.9 ± 0.08  | <0.78             | 3.7 ± 0.26          | 0.4 ± 0.06   | 293 ± 5.5    | 9.0 ± 0.75                    | 37.0 ± 1.75                              |
| AH\( ^{200} \) | 2.4 ± 0.17  | <0.78             | 4.2 ± 1.1           | 0.3 ± 0.01   | 342 ± 4.2    | 7.6 ± 1.12                    | 32.3 ± 1.26                              |

\( ^a \) c.u./mg, enzyme unit/mg protein.  
\( ^b \) k/mg, first-order kinetic constant/mg protein.  
\( ^c \) nm/min/mg = nmole/min/mg protein.

**RESULTS**

**BP and DMBA Resistance**—We developed a series of BP-resistant human breast cancer MCF-7 cells by continuous exposure to BP. The BP concentration which inhibited cell growth by 50% (IC\( _{50} \)) in WT, AH\( ^{40} \), AH\( ^{100} \), and AH\( ^{200} \) was 0.6 ± 0.07, 20 ± 0.67, 85 ± 7.3, and 164 ± 6.9 \( \mu \)M, respectively (Fig. 2). The resistant cell lines showed no increase in QR activity, while the WT, AHR40, AHR100, and AHR200 cells showed no increase in G6PD activity. The amount of G6PD mRNA was 1.0, 2.5, 3.7, and 5.6 in WT, AHR40, AHR100, and AHR200, respectively (Table I).

**G6PD Activity**—We measured G6PD activity under \( V_{\text{max}} \) conditions in cell cytosol and found that the activity of G6PD in WT, AH\( ^{40} \), AH\( ^{100} \), and AH\( ^{200} \) was 41.2 ± 2.65, 85.5 ± 3.96, 126 ± 8.16, and 165 ± 12.2 nmol/min/mg protein, respectively (Fig. 3A). We further examined the enzyme kinetics of G6PD in WT and AH-resistant cells. Results are illustrated in the form of a Hanes-Woolf plot (Fig. 3B) and reveal an increase in \( V_{\text{max}} \) with increasing NADP\(^+\) concentrations, while the \( K_m \) value remained unchanged. In contrast, there was no change in 6-phosphogluconate dehydrogenase activity in the resistant cells (Table I).

**Detoxification Enzyme Activities**—The activities of glutathione peroxidase, GST, GST-\( \tau \), and catalase were not significantly changed in the AH\( ^{8} \) cell lines compared with the WT (Table I). The level of QR activity was increased in AH\( ^{100} \) and AH\( ^{200} \) cells compared with the WT, but the AH\( ^{40} \) cells did not have any significant increase in QR activity (Table I).

**G6PD Expression**—The expression of G6PD in the cell lines was examined at the protein and mRNA levels. The amount of G6PD protein, as measured by Western blot, the relative amounts of G6PD mRNA, and the levels of G6PD promoter were determined by densitometry and normalized to actin protein. This experiment was repeated once with different cell preparations with similar results.

**G6PD-Promoter-Controlled Transcription**—G6PD promoter-driven transcription was examined by transiently transfecting...
WT and AHR cells with a luciferase reporter vector containing the full-length G6PD promoter. As shown in Fig. 6, there was no increase in luciferase transcription in AHR cells when compared with WT. In order to demonstrate the responsiveness of this vector to known inducers of G6PD transcription, we treated transfected WT cells with diamide, a well characterized inducer of G6PD transcription (11). In agreement with previous results, diamide caused a 2-fold increase in G6PD transcription (Fig. 6). As an additional positive control, transfected WT cells were deprived of glucose and then refed, a treatment which is known to induce G6PD transcription. This caused an 8-fold increase in G6PD-promoter controlled transcription (data not shown).

**G6PD mRNA Stability**—The stability of G6PD mRNA in the WT and AHR cells was determined by an actinomycin D chase experiment using RT-PCR. There was a greater amount of mRNA in the AHR cells compared with WT cells when compared with WT. In order to demonstrate the responsiveness of this vector to known inducers of G6PD transcription, we treated transfected WT cells with diamide, a well characterized inducer of G6PD transcription (11). In agreement with previous results, diamide caused a 2-fold increase in G6PD transcription (Fig. 6). As an additional positive control, transfected WT cells were deprived of glucose and then refed, a treatment which is known to induce G6PD transcription. This caused an 8-fold increase in G6PD-promoter controlled transcription (data not shown).

**Glutathione Concentrations**—GSH levels were measured in the cell lines. There was an increase in GSH with increasing AH resistance (Fig. 8).

**DISCUSSION**

Environmental carcinogens such as BP exert their deleterious effects in at least two ways: epoxide metabolites generated by Phase 1 enzymes such as cytochrome P450 1A1 are highly electrophilic and bind nucleophilic residues in DNA, forming adducts (29). Second, the epoxide can enter quinone/semiquinone redox cycling, generating free radicals which can oxidize DNA (30). The end result of both mechanisms is the introduction of specific mutations which may lead to cellular transformation. Cells have developed a number of defense mechanisms to deal with xenobiotics. These include the Phase 2 enzymes, which conjugate AH metabolites, forming relatively inert compounds that are easily excreted. Cells also synthesize glutathione, a cysteine-containing tripeptide ubiquitously found in eukaryotic cells at concentrations of 0.5 to 10 mM. GSH has reducing and nucleophilic properties that make it a key element in cellular protection from oxidative damage. Furthermore, GSH is a cofactor of GST, which is responsible for detoxification of xenobiotics. Regeneration of GSH from oxidized glutathione by glutathione reductase, as well as many of the Phase 2 enzymes such as QR, requires NADPH as a co-factor. The intracellular level of NADPH is therefore a critical factor in carcinogen detoxification. The enzyme that regulates NADPH synthesis, G6PD, is now recognized as an important and highly regulated enzyme (31). Despite the central role of the PPP in supplying NADPH and hence, GSH, for carcinogen detoxification, little is known about the modulation of this pathway by exposure to xenobiotics. We hypothesized that modulation of G6PD is important for detoxification mechanisms because of its role in providing reducing potential for the regeneration of GSH. To test this hypothesis, we developed a series of carcinogen-resistant cells from the carcinogen-sensitive human breast cancer MCF-7 cells. MCF-7 cells have been used extensively as a model for the study of G6PD by our laboratory (32) and others (33, 34). We examined the following two questions. 1) Do cells modulate G6PD to adapt to carcinogen exposure? 2) How do cells carry out this modulation?

We found that the enzymatic activity of G6PD was significantly increased in AH-resistant cells compared with the WT cell, and that this increase in activity was positively correlated with increasing resistance (Fig. 3). To test whether this increase in specific activity was the result of changes in the enzyme/substrate interaction, we performed kinetic analysis by Hanes-Woolf plot. This analysis showed that there was an increase in the $V_{max}$ of G6PD in AHR compared with the WT,
but no change in $K_m$, indicating that the enzymatic mechanism of action is similar in the different cell lines. It has been previously documented that exogenous chemicals which cause oxidative stress, such as diquat (10) or diamide (11), enhance G6PD activity, but this is the first observation, to our knowledge, of an increase in G6PD activity associated with carcinogen exposure.

Because the above result indicates an increase in the amount of G6PD in resistant cells, we examined protein levels by Western blot. As shown in Fig. 4, there was an increase in the enzyme in AHR that was correlated with the increase in AH resistance. This was supported by Northern blot analysis (Fig. 5) which showed an increase in G6PD mRNA correlated with AH resistance. These results clearly demonstrate that the resistant cell lines have an increase in G6PD expression that varies with the level of resistance. Interestingly, there was no change in the level of transcriptional activity of a reporter vector containing the G6PD promoter in AHR cells compared with WT (Fig. 6). Treatments that are known to increase G6PD transcription, such as glucose deprivation/refeeding and diamide, increased transcription in transfected WT cells, demonstrating the appropriate responsiveness of this vector. These results indicate that the increase in G6PD expression that is associated with AH resistance is not the result of an increased rate of G6PD transcription. This leaves the possibility of a post-transcriptional mechanism of modulation. This was examined by treating the WT and AH R200 cells with actinomycin D to inhibit transcription. The stability of G6PD mRNA was assessed using RT-PCR (Fig. 7). This experiment showed that the rate of G6PD mRNA degradation was faster in the WT cells than in the AH R200 cells, indicating that the mRNA in the AH R200 cells was more stable. Thus, the increase in G6PD expression in AH R cells is due to a post-transcriptional stabilization of G6PD mRNA. There is precedent for this type of post-transcriptional control of G6PD expression. Prostko et al. (35) found that a high-carbohydrate diet increased G6PD mRNA stability, resulting in an increase in enzyme activity. Other post-transcriptional modes of regulation have also been demonstrated for G6PD expression (14, 15, 36). Although mRNA stability plays a major role in the determination of gene expression, the regulation of mRNA stability is poorly understood, and the mechanism which modulates G6PD mRNA stability remains to be studied.

Since it is well established that the activity of G6PD is directly linked with the maintenance of GSH levels, we examined the cell lines for GSH. As shown in Fig. 8, there is an increase in GSH levels that corresponds with the increase in AH resistance. This was not due to increases in glutathione reductase activity, which was unchanged in the AHR cell lines (Table I). The increase in G6PD activity therefore has functional significance for carcinogen detoxification.

These results do not prove that the increase in G6PD expression is the sole or definitive mechanism responsible for AH resistance. We have examined several other mechanisms which may contribute to resistance. Although there was no significant increase in total GST, GST-π, glutathione peroxidase, or catalase activities in resistant cells, there was an increase in QR activity in the AH R100 and AH R200 cell lines (Table I), although not in the AH R40 cell line, indicating that increased QR may be involved in higher-stage resistance. Furthermore, we and others (37) have demonstrated that BP resistance in vitro is also associated with changes in aryl hydrocarbon receptor activity, resulting in impaired induction of cytochrome P450 1A1, a major AH activating enzyme in these cells. This results in a decrease in AH activation. This suggests that both carcinogen

\[ \text{FIG. 7. G6PD mRNA stability of WT and AH R200 cells.} \]

\[ \text{WT and AH R200 cells were treated with the RNA synthesis inhibitor actinomycin D for 8 h to stop transcription. Total RNA was isolated at 8, 16, 24, and 48 h after the addition of actinomycin D, and G6PD mRNA was determined by RT-PCR as described. The assay was done in duplicate and repeated once with similar results (closed squares, WT; open diamonds, AH R200).} \]

\[ \text{FIG. 8. Reduced glutathione concentrations in WT and AH R cells.} \]

\[ \text{GSH levels in WT and AH R cells were determined as described under "Experimental Procedures." The enzyme activity was determined in duplicate for each experiment and the values are given as mean ± S.E. of three determinations.} \]

\[ \text{‡ G. C. Yeh and H. P. Ciolino, unpublished data.} \]
activation and detoxification mechanisms work in concert for cellular defense against carcinogen insult.

These results clearly demonstrate that there is an increase in the expression and activity of G6PD in our AH cells that is correlated with AH resistance, and that this modulation is the result of a post-transcriptional regulation. The major site of AH metabolism and detoxification is the liver, which expresses high levels of G6PD. Although not specifically addressed in the current experiments, similar induction of hepatic G6PD expression and activity upon long-term exposure to AH would result in an increase in the detoxification capacity of the liver and provide an additional level of AH resistance. Thus, modulation of G6PD expression may be an important part of the adaptation of cells to long-term exposure to AH. This is a heretofore unrecognized cellular defense mechanism against carcinogen exposure.

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