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Cytosolic enzymes from rat tissues that activate the cooked meat mutagen metabolite N-Hydroxyamino-1-methyl-6-phenylimidazo[*4,5-b*]pyridine (N-OH-PhIP)

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Abstract

Heterocyclic amines are formed during the cooking of foods rich in protein and can be metabolically converted into cytotoxic and mutagenic compounds. These "cooked-food mutagens" constitute a potential health hazard because DNA damage arising from dietary exposure to heterocyclic amines can modify cell genomes and thereby affect future organ function. To determine enzymes responsible for heterocyclic amine processing in mammalian tissues, we performed studies to measure genotoxic activation of the N-hydroxy form of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) –a common dietary mutagen. O-Acetyltransferase, sulfotransferase, kinase, and amino-acyl synthetase activities were assayed using substrate-specific reactions and cytosolic enzymes from newborn and adult rat heart, liver, spleen, kidney, brain, lung, and skeletal muscle. The resultant enzyme-specific DNA adduct formation was quantified via ^{32}P-postlabeling techniques. In biochemical assays with rat tissue cytosolic proteins, O-acetyltransferases were the enzymes most responsible for N-hydroxy-PhIP (N-OH-PhIP) activation. Compared to O-acetyltransferase activation, there was significantly less kinase activity and even lesser amounts of sulfotransferase activity. Proyl-tRNA synthetase activation of N-OH-PhIP was not detected. Comparing newborn rat tissues, the highest level of O-acetyltransferase mutagen activation was observed for neonatal heart tissue with activities ranked in the order of heart > kidney > lung > liver > skeletal muscle > brain > spleen. Enzymes from cultured neonatal myocytes displayed high O-acetyltransferase activities, similar to that observed for whole newborn heart. This tissue specificity suggests that neonatal cardiac myocytes might be at greater risk for damage from dietary heterocyclic amine mutagens than some other cell types. However, cytosolic enzymes from adult rat tissues exhibited a different O-acetyltransferase activation profile, such that liver > muscle > spleen > kidney > lung > brain > heart. These results demonstrated that enzymes involved in catalyzing PhIP-DNA adduct formation varied substantially in activity between tissues and in some tissues, changed significantly during development and aging. The results further suggest that O-acetyltransferases are the primary activators of N-OH-PhIP in rat tissues.

Keywords: Cooked meat mutagens; Heterocyclic amines; PhIP; DNA damage; Rat tissues; Phase II activation; Carcinogen metabolism

1. Introduction

To understand how foods sustain optimum health or contribute to human disease, the interactions between dietary components and human genomes need to be elucidated. One class of potentially harmful compounds present in the diet of industrialized societies is heterocyclic amines. Heterocyclic amines are produced during cooking of meats and fish over high heat [1,2]. These compounds cause mutations, and many are carcinogenic in animal models [3–5]. Heterocyclic amines are also enriched in pan gravies [6], and epidemiological studies have revealed that individuals who regularly consume well-done meats and heavily browned gravies are at greater risk for developing some types of cancers [7–9]. One of the most abundant heterocyclic amines in cooked meats is 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) [9], and evidence has been presented for a possible role for PhIP in human carcinogenesis [10–12].

The ability to covalently modify DNA is an integral part of heterocyclic amine-initiated carcinogenesis. When fed to Fischer-344 (F344) rats, PhIP was found widely distributed

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in the tissues, and PhIP-DNA adducts were formed in all organs examined [12–18]. When incorporated in the feed, PhIP has been shown to induce mammary gland tumors in female F344 rats, whereas prostate and colon tumors were induced in male F344 rats [11,12,19–21]. However, there are other biological systems that participate in targeting a carcinogen to an organ, and DNA adducts levels are only one component in the overall process of tumorigenesis. In addition to being linked with tumor incidence, PhIP might also be involved with tissue degeneration in some target organs wherein PhIP-DNA adducts are formed at high levels in response to dietary exposure. For example, studies have shown that heart tissue is particularly prone to suffer more PhIP-related DNA damage than several other rodent tissues [13,17]. Therefore, PhIP-DNA adducts may be cardiotoxic, and it has been suggested that prolonged exposure to PhIP and related compounds may be involved in cardiac degeneration in susceptible individuals [22–25].

Like other heterocyclic amines, PhIP requires metabolic activation to exert its biological effects. Metabolic activation proceeds by a two-step biotransformation. The extracyclic amino group is first oxidized by cytochrome P450-enzymes (phase I activation) that are generally most active in liver tissue [26–29]. The N-hydroxylated metabolite of PhIP (N-OH-PhIP) may then be converted by esterification reactions (phase II activation) to highly reactive electrophilic compounds that covalently bind DNA [14]. Several phase II activation enzymes, such as O-acetyltransferases and sulfotransferases, have been described that result in acetylation and sulfation of N-OH-PhIP, respectively [5,30, 31]. Less well-characterized activation pathways that involve phosphorylation and amino acid acylation have also been considered [5,32–34].

Since levels of genome damage might be directly related to organ-specific differences in metabolic processing of carcinogens, we have assayed for the enzymes believed to be responsible for converting the intermediate mutagen N-OH-PhIP to more reactive proximal or ultimate mutagens that add on to DNA. To approximate the biochemical basis for differences in adduct level between tissues, the number of PhIP molecules covalently attached to DNA as a function of substrate-dependent reactions using cytosolic enzymes from seven different tissues of newborn and adult F344 rats have been determined. Cultured neonatal rat cardiac myocytes, which are frequently used as a model system to study toxic chemicals, were also studied.

2. Materials and methods

2.1. Chemicals

PhIP was purchased from Toronto Research Chemicals (North York, Ontario). N-hydroxy-PhIP (N-OH-PhIP) was obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository at the Midwest Research Institute (Kansas City, MO) and was greater than 98% pure. [γ-^{32}P]-ATP (>4,000 Ci/mmol) was from ICN (Irvine, CA). Cell culture media, reagents, and Proteinase K were supplied by Gibco BRL (Rockville, MD). Polyethyleneimine (PEI) cellulose thin layer sheets were from Alltech (Deefield, IL). T4 polynucleotide kinase was obtained from MBI-Fermentas (Amherst, NY). L-Proline, adenosine 3′-phosphate 5′phosphosulfate (PAPS), acetyl coenzyme A (AcCoA), ATP, Micrococcal nuclease, spleen phosphodiesterase (Type II), calf thymus DNA (type I), and all other chemicals were from Sigma (St. Louis, MO).

2.2. Animals and cell culture

2.2.1. Rats

Adult female Fischer-344 rats (150–165 g, 10- to 11-weeks-old) were acquired from Charles River Laboratories (Willminton, MA). Rats were housed one per polycarbonate cage in climate controlled rooms. Purina 5001 rat chow and water were supplied ad libitum.

2.2.2. Cardiac myocyte cell culture

Myocytes were isolated from neonatal, 1- to 2-day-old, F344 rat pups. Hearts were removed, trimmed of connective tissue, finely minced with scissors, and immediately placed in the nominally calcium-free dissociation buffer of Simpson and Savion [35], containing 0.005% DNase I, 0.1% collagenase II, 0.1% trypsin, and 25 U-μg/ml Penicillin-Streptomycin (Pen-Strep). Myocytes were dissociated by repeated cycles of 10-min digestions with slow stirring, then cells were sedimented at 300 x g for 5 min, 4°C. This process was repeated until the tissue was completely dissociated. Cells were resuspended in M199 medium, supplemented with 4.17 mM $NaHCO_3$, 1.5 μM Vitamin B_{12}, 0.1 μM $ZnSO_4$, and 0.12 μM $CuSO_4$ (supplemented M199 media) containing 10% calf serum and 25 U-μg/ml Pen-Strep. Since non-myocytes attach to the plastic substrata more rapidly than myocytes, a selective 1-hr attachment was carried out to remove non-myocytes [35]. Primary cultures of neonatal rat cardiac myocytes were obtained by allowing myocytes to attach for a minimum of 4 hr, unattached cells were removed, attached cells were washed with warm PBS, and M199 media, supplemented with 10^{-5} M bromodeoxyuridine (BrdU), was added. Cardiac cells were cultured in supplemented M199 media containing 5% calf serum and 50 U-μg/ml Pen-Strep in a humidified 95% air/5% CO_2 incubator at 37°C. Cell numbers and morphology were examined with a Nikon model M inversion microscope. Myocytes are clearly identifiable as larger cells that beat [35]. Myocytes were cultured for 3 days or longer before they were used in experiments.

2.2.3. Cardiac non-myocyte cell culture

After the first 1-hr rapid attachment, unattached cells were removed, and supplemented M199 media with 5% calf serum was added to the attached cells. These cells were

allowed to proliferate for 24 to 48 hr, then media containing 10^{-5} M BrdU was added and the cultures are maintained in BrdU thereafter. BrdU will suppress further cell division [35]. Based on size and morphology, these cells were more than 90% fibroblasts.

2.3. Preparation of cytosols

2.3.1. Tissues

Fresh rat tissue samples were placed in ice-cold PBS, pH 7.4, minced with scissors, and rinsed several times to remove excess blood and fat. The tissue was homogenized in 0.25 M sucrose containing 10 mM triethanolamine-HCl, pH 7.4, and 5 mM 2-mercaptoethanol **(**STM**)** using a Brinkman Polytron (2 separate pulses of 5 W for 10 sec). Two vol of homogenization buffer per g wet weight of tissue was used. Homogenates were immediately subjected to centrifugation at 105,000 x g for 1 hr at 4°C, and supernatants were assayed for protein concentration and enzyme activity.

2.3.2. Cultured cells

Cell cultures were washed with warm Hank's Balanced Salt Solution, containing 1 mM EDTA, then treated with trypsin to release them from the culture dish. Cells were sedimented at 870 x g for 10 min at 4°C using 15-ml conical tubes. The supernatant was decanted, and residual liquid was blotted off. The pellet was suspended in 2 ml of ice-cold STM, and cells were disrupted with a Polytron (5 W, 1 sec). The homogenate was subjected to centrifugation at 105,000 x g for 1 hr at 4°C. The supernatant was immediately assayed for protein concentration and enzyme activity. Protein concentrations were determined by the method of Bradford [36].

2.4. Esterification activation assays

To maximize the use of cytosolic proteins, activation assays were scaled to 200 μl total volume. All assays were performed for 30 min at 37°C in argon-saturated buffers. Reactions were assembled on ice in tightly sealed tubes with argon saturation of incubation mixture and head-space. Reactions were initiated by the addition of 20 μM N-OH-PhIP in 5 μl of DMSO-ethanol, 4:1 and placing them at 37°C. The DNA-binding assays were terminated by the addition of 2 volume of water-saturated n-butanol, and DNA was isolated as described below. In initial studies, it was determined that conditions were saturating for substrate and first order with respect to protein concentration. Background binding of N-OH-PhIP to DNA was estimated by omitting cytosolic proteins from the incubation mixture, and these values were subtracted from those obtained in enzyme-dependent reactions.

2.4.1. Acetyltransferase assay

Acetyl-CoA-dependent O-acetyltransferase activity was determined as reported by Turesky et al. [28] using 50 mM pyrophosphate buffer (pH 7.4) containing 1 mM DTT, 1 mM AcCoA, 2 mg/ml calf thymus DNA, 1 mg/ml cytosolic protein, 0.1 mM EDTA, and 20 μM of N-OH-PhIP substrate.

2.4.2. Sulfotransferase assay

3′-Phosphate-adenosine-5′ phosphosulfate (PAPS)-dependent sulfotransferase activation of N-hydroxylamine heterocyclic compounds was assayed by a method modified from Lin et al. [33]. Incubation mixtures consisted of 50 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 2 mg/ml calf thymus DNA, 1 mg/ml cytosolic protein, and 0.2 mM PAPS.

2.4.3. Kinase and tRNA synthetase assays

These DNA binding assays are from Lin et al., 1995, wherein cytosolic L-proline-dependent and ATP-dependent tRNA synthetase and ATP-dependent kinase activities were determined using methods similar to those of Davis et al. [32]. Incubation mixtures contained 50 mM potassium N,N′-bis(2-hydroxyethyl)glycine (Bicine) buffer, pH 8.0, 2 mg/ml calf thymus DNA, 1 mM DTT, 3 mM magnesium acetate, 1 mM L-proline and/or 1 mM ATP, 20 μM N-OH-PhIP, and 1 mg/ml of cytosolic protein. There were three assays: (a) *cytosol plus L-proline for* L-proline-dependent activation, (b) cytosol plus ATP for ATP-dependent kinase activation, and (c) cytosol plus proline and ATP for ATP-dependent proyl-tRNA synthetase activation.

2.5. DNA isolation

Reactions were terminated by the addition of 2 vol of water-saturated n-butanol. The aqueous phases were made 300 mM sodium acetate (1/10 vol of 3 M stock), mixed well, and extracted twice with buffer-saturated phenol-chloroform (1:1, vol/vol). The DNA was precipitated from the aqueous phase with 2.5 vol of cold ethanol. Precipitated DNAs were dissolved in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. The concentrations of DNA recovered from the reactions were determined from A_{260nm} absorbances.

2.6. PhIP-DNA adduct determination by ^{32}P-postlabeling

Postlabeling analysis of DNA adducts was carried out according to the ATP-deficient, intensification methods of Randerath et al. [37].

2.6.1. Digestion of DNA

Samples corresponding to 10 μg of DNA were digested in a 20 μl total volume of 10 mM succinate, 5 mM $CaCl_2$, pH 6.0, with 5 μg each of spleen phosphodiesterase and Micrococcal nuclease. Standard incubation was 37°C for 3 hr.

2.6.2. ^{32}P-Labeling of DNA digests

Samples corresponding to 5 μg of DNA digests were dried in a SpeedVac concentrator. Nucleotides were incubated with [γ-^{32}P]ATP (4500 Ci/mmol, 0.035 nmol, 13.50

μl of 10 μCi/μl) in 10 μl kinase buffer (0.3 M Tris-HCl, pH 9.5, 0.1 M $MgCl_2$, 0.1 M DTT, and 0.01 M spermidine) with 2.5 units of polynucleotide kinase for 30 min at 37°C.

2.6.3. Thin layer chromatography (TLC) and adduct mapping

Following labeling, normal nucleotides were separated from modified nucleotides by TLC development of samples on PEI-cellulose sheets with 1.7 M sodium phosphate, pH 6.0. Modified nucleotides remained at the origin while normal nucleotides migrated away from the origin [37]. The modified nucleotides were contact-transferred to another PEI-cellulose sheet and developed with the following solvent systems: (D1, first dimension) 3.6 M lithium formate - 6.8 M urea, pH 3.5; (D2, second dimension at right angles to D1) 0.6 M sodium phosphate - 0.5 M Tris-HCl −6.5 M urea, pH 8.2; followed by redevelopment in D2 with 1.7 M sodium phosphate buffer to reduce background radioactivity. Adducts were detected by autoradiography on X-ray film with intensifying screens at -70°C. Using this TLC separation system, we have observed one abundant and two minor adducts in mammalian DNA isolated from cell cultures exposed to N-OH-PhIP [34]. No adducts above background have been noted for experiments using the parent compounds (not shown). To determine total nucleotide labeling for each sample, 1 μl of the labeling reaction was removed and rapidly mixed with 59 μl of 10 mM Tris-HCl, 1 mM EDTA, pH 9.5. Five μL aliquots of this dilution were applied to a TLC-sheet, and nucleotides were separated by chromatography with 0.8 M ammonium formate, pH 3.5.

2.6.4. Adduct Quantification

Adducts were quantified as described in previous work [37,38]. Radioactivity associated with an adduct was determined by aligning a TLC sheet with its corresponding autoradiogram; the various adducts were marked; scissors were used to cut out the adduct which was then place in a scintillation vial for Cerenkov counting. The same chromatographic areas were removed from negative control reaction, e.g., minus cofactor or protein, for use in background subtraction. The level of covalently modified nucleotide is determined from **relative adduct labeling** (RAL) corrected for standard leveling conditions of ATP excess by the technique of Schut and Herzog [38]. RAL was calculated from the formula: RAL = cpm in adduct - cpm of background/cpm in total nucleotides. Mutagen-DNA binding values are expressed as PhIP-DNA adducts per total nucleotides.

2.7. Statistical analysis

Data are expressed as the mean plus and minus the standard deviation. Significance was determined by pairwise comparisons made using the Student's t-test method.

3. Results

^{32}P-postlabeling methods [37,38] were used to measure the enzymatic conversion of N-OH-PhIP to PhIP-DNA adducts as mediated by cytosolic proteins from rat tissues. Enzyme-specific DNA binding assays were used instead of direct measurements because the immediate products of these activation reactions (e.g., N-acetoxy-PhIP produced by O-acetyltransferases) are unstable and rather rapidly decompose under the conditions of their formation [39]. DNA therefore acts as a binding acceptor of reactive PhIP electrophiles generated in phase II reactions. Autoradiograms of PhIP-DNA adducts formed by AcCoA-dependent O-acetyltransferases reactions from 2-day-old rat heart, kidney, lung, liver, thigh muscle, and brain are shown in Figure 1. Adducts formed by O-acetyltransferases from the same tissues of adult female rats are shown in Figure 2. The PhIP-DNA adducts are represented as spots on autoradiograms of x-ray film exposed to thin-layer-chromatography sheets. The number of adducts commonly observed under our labeling conditions was three, which is typical of ^{32}P-postlabeling adduct patterns induced by PhIP in vitro and in vivo [14–18,23,34]. The major DNA adduct formed with N-OH-PhIP has been identified as N-deoxyguanosine-8-yl)-PhIP [50] (dG-C8-PhIP). Adducts were assigned numbers corresponding to results of previous studies [34,38]. In some cases, a minor fourth spot was observed (see for instance Figure 1-A). This additional spot was not reproducible and has been attributed to incomplete nuclease digestion [40]. Acetyltransferases from all tissues gave similar adduct patterns. The results obtained with pancreas tissue are not shown but produced a similar pattern of adducts. This adduct fingerprint was also observed in sulfotransferase (PAPS-dependent) and kinase (ATP-dependent) reactions as shown in Figure 3 for newborn rat liver tissue. We detected no L-proline activation above background or L-proyl-tRNA synthetase activation (L-proline- and ATP-dependent) above ATP-dependent activation alone which suggest that in rat tissues synthetase activity is absent or below detection limits. The ^{32}P-postlabeling results indicate that in rat tissues O-acetyltransferases, sulfotransferases, and kinases have the ability to biotransform N-OH-PhIP to generate PhIP-DNA adducts, and these adducts yield a characteristic adduct chromatographic fingerprint.

To determine the potential genotoxic contribution of different phase II activation pathways, PhIP-DNA adducts were quantified, and the results for different rat tissue cytosolic proteins are shown in Table 1. Relative O-acetyltransferase, sulfotransferase and kinase enzyme activities presented in Table 1 represent total PhIP-DNA adduct formation, i.e., the sum of all adducts formed per mg protein in a 30 min reaction. Phase II activation of N-OH-PhIP varied considerably between enzymes. However, the data show that O-acetyltransferases were the major esterification activity for cytosolic proteins from all rat tissues tested. Sulfotransferases displayed minimal activity in facilitating

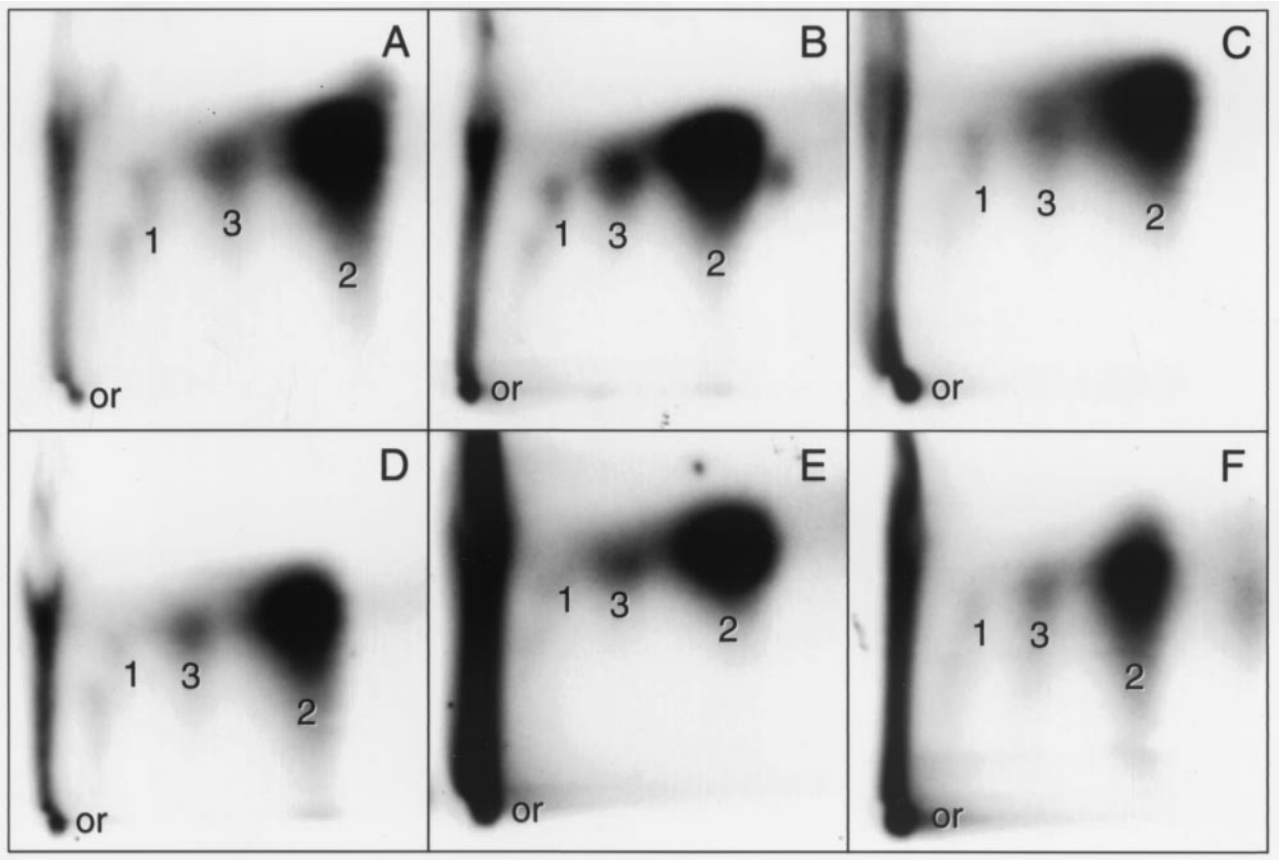

Fig. 1. PhIP-DNA adducts generated by O-acetyltransferase enzymes from newborn rat tissues. Adducts were separated by multi-dimensional, thin-layer chromatography on PEI sheets, and chromatograms were exposed to x-ray film for varying times at –70°C. Adducts are numbered according to previously established patterns (34). Tissues and autoradiography times were: (A) heart tissue, 6 hr; (B) kidney tissue, 8 hr; (C) lung tissue, 8 hr; (D) liver tissue, 6 hr; (E) thigh muscle, 16 hr, and; (F) brain tissue, 24 hr. All reactions contained 1 mg/ml cytosolic protein, 20 μM N-HO-PhIP, and 1 mM AcCoA incubated with 2 mg/ml of calf thymus DNA for 30 min at 37°C. Reactions without AcCoA showed no adducts. Or indicates the chromatographic origin.

PhIP-DNA adduct formation, and in most instances, adduct levels for PAPS-dependent reactions were not significantly different from background, and in fact, could not be detected for newborn heart, kidney, and brain proteins. On the other hand, kinases exhibited intermediate activation of N-OH-PhIP –more than sulfotransferases but less than O-acetyltransferases. Kinase activity, moreover, varied from close to background levels of binding for adult thigh muscle to levels some 140-times higher ascertained for rat brain kinases. Less extreme variation was observed with O-acetyltransferases. The greatest difference was 8-fold between the O-acetyltransferase activities of neonatal tissue heart cytosols and spleen cytosols. In addition to tissue differences, N-OH-PhIP phase II enzyme activation also varied with age. Newborn rat heart tissue exhibited the highest mutagen O-acetyltransferase activity out of the seven tissues surveyed. However, the inverse situation was found for adult heart tissues where heart O-acetyltransferase activity was the lowest of the seven tissues examined. Another large age-related difference was noted for spleen tissue where the newborn activation was only 15% of the adult value. The results comparing different phase II activating enzymes among various tissues suggest that O-acetyltransferases have the greatest potential to metabolize N-OH-PhIP to DNA reactive compounds in many different types of rat cells. However, as indicated by the rather large tissue-dependent and age-related differences in total adduct formation, the differentiated state of each cell plays a major role determining the composition and amounts of N-OH-PhIP activating enzymes.

One of the initial objectives of this study was to investigate PhIP metabolism in heart cells because prior studies had indicated that myocardium might be a target tissue for this heterocyclic amine [22–25]. Indeed, the data shown in Table 1 supported this notion owing to the very high O-acetyltransferase activity of newborn heart cytosol. Therefore to examine heart N-OH-PhIP metabolism in greater detail, we studied mutagen activation using cultured neonatal rat cardiac cells which is a well-established model system for the study of drug metabolism and toxicity in myocytes [41]. For comparison, non-myocytes from rat heart tissue were also cultured. Preparing cytosol from the two types of cultures, we assayed for phase II activation enzymes. The results revealed that rat heart cells contained O-acetyltransferases and kinases that were capable of processing N-OH-PhIP to PhIP-DNA adducts. For example,

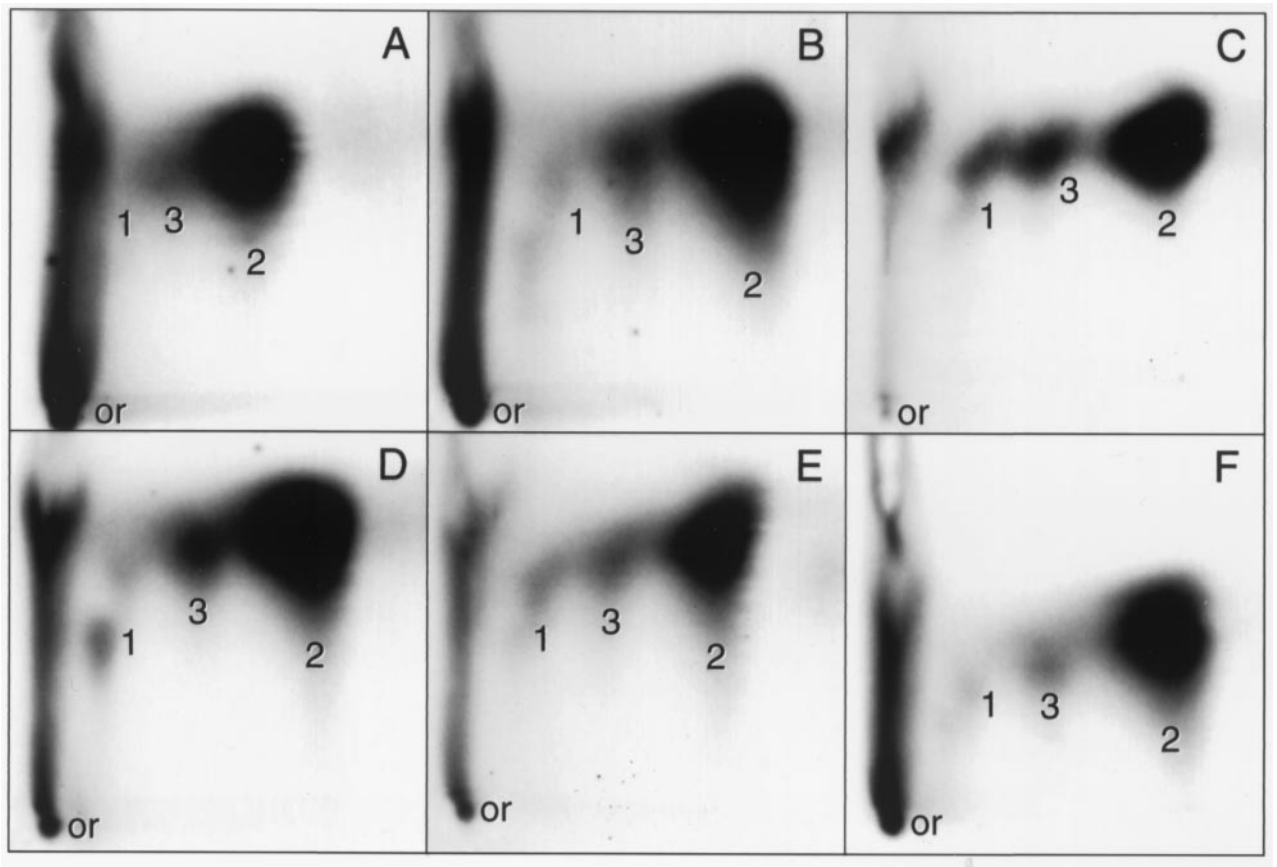

Fig. 2. PhIP-DNA adducts produced by O-acetyltransferase enzymes from adult female F334 rat tissues. Reaction and chromatography conditions were the same as described in the legend to Figure 1. Tissues and autoradiography times were: (A) heart tissue, 16 hr; (B) kidney tissue, 16 hr; (C) lung tissue, 6 hr; (D) liver tissue, 6 hr; (E) thigh muscle, 6 hr, and; (F) brain tissue, 8 hr. Or is the origin.

Figure 4 shows acetyltransferase and kinase ^{32}P-postlabeling results using cardiac myocyte proteins (Figures 4-A and 4-B, respectively) and non-myocyte proteins (Figures 4-C and 4-D). In agreement with the data obtained with whole heart enzymes, cultured cardiac myocytes and non-myocytes displayed no detectable sulfotransferase N-OH-PhIP activation. Furthermore, the levels of activation determined for cultured myocyte kinases and O-acetyltransferases closely matched those of heart tissue (Figure 5). Both kinase and O-acetyltransferase enzymes isolated from non-myocyte cultures showed significantly higher levels of activation, and in fact, exhibited the highest activation potential of any tissue or cell tested. As described under methods, this latter result might be due to in vitro proliferation of non-myocytes, such as fibroblasts. However, the experiments with primary cultures of neonatal rat cardiac myocytes suggest that this model system may be appropriate for studying the metabolic processing of cooked meat mutagens, such as PhIP.

4. Discussion

In these studies, we have attempted to gain some insight into PhIP-induced DNA damage in vivo by studying phase II activation enzymes from different rat tissues. Several groups have reported that rats given PhIP in their feed will incur DNA damage in all organs. Tissues tested thus far include stomach, colon, muscle, small intestine, testis, bladder, kidney, liver, lung, spleen, heart, pancreas, brain, mammary gland and prostate [11–18]. However, these animal studies did not demonstrate what enzymes were converting PhIP and PhIP metabolites to DNA-modifying compounds in these tissues. It is generally conceded that most phase I activation of PhIP to N-OH-PhIP occurs in hepatocytes by cytochrome P450 1A1 and 1A2 enzymes [14,27,28]. Here we found that every tissue examined possessed cytosolic O-acetyltransferases and kinases capable of activating N-OH-PhIP. On the other hand, sulfotransferase activities were very low or absent in various rat tissues, and proyl-tRNA synthetase activity was not detected. In vitro DNA binding assays clearly showed that O-acetyltransferases had the highest activity of phase II enzymes in the seven different rat tissues tested. If O-acetyltransferases are indeed the major activation enzymes in cells, then O-acetyltransferase activation should correlate with tissue adduct levels. For animals, however, PhIP-DNA adduct levels depend upon many experimental variables, such as type, dose, and duration of exposure and may depend on biological variables

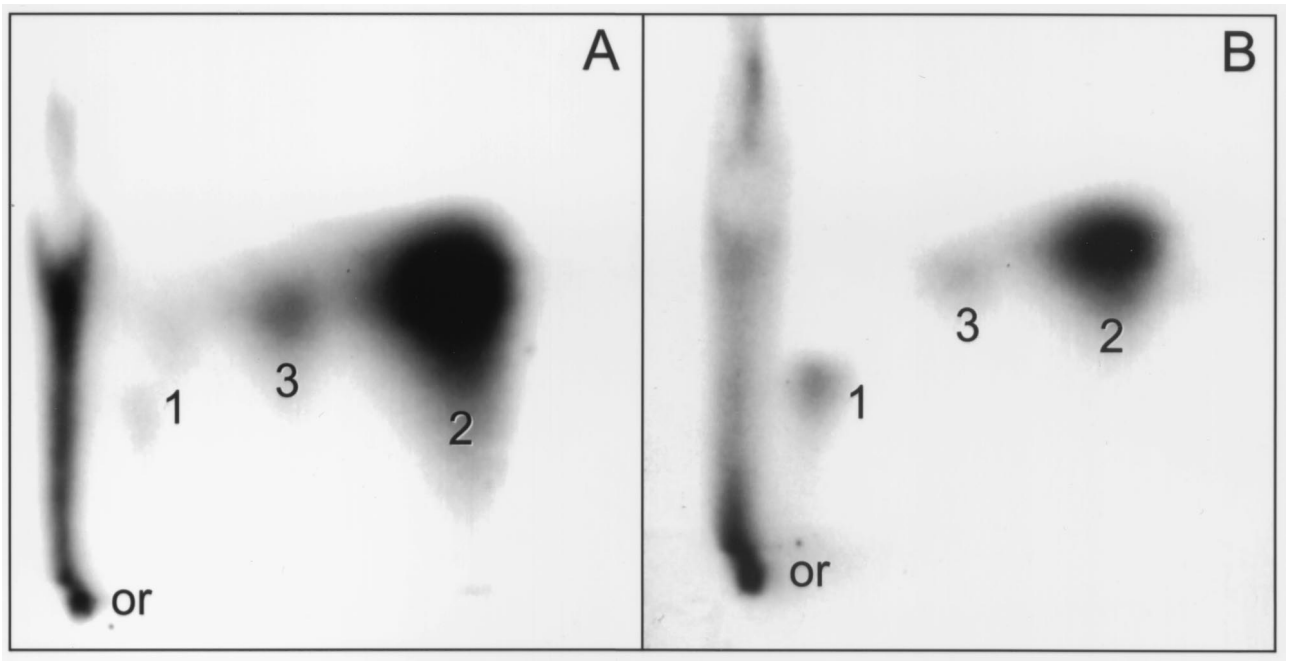

Fig. 3. PhIP-DNA adducts produced by newborn rat liver kinases (A) and sulfotransferases (B). Reaction and chromatography conditions were the same as described in the legend to Figure 1 except the substrates were 1 mM ATP and 1 mM PAPS for kinases and sulfotransferases, respectively. Autoradiography times were 16 hr (A) and 48 hr (B).

such as age, sex and strain of rodent. Whether or not the tissue is composed of proliferating cells or postmitotic cells is an additional concern. Nonetheless, results from multiple studies, analyzing young rats less than 7-weeks-old following multiple PhIP exposures over 2 to 4 weeks, have shown a consensus in that heart is among the top tissues in PhIP-DNA adduct abundance [13,16–18]. Cardiac tissue therefore appears to be an organ of preferential mutagen activation or accumulation in young animals [22,23], and this conclusion fits well the N-OH-PhIP activation shown in Table 1 for newborn heart. Cardiac tissue from adult female rats presented a different picture where heart had a decreased ability to activate N-OH-PhIP and in fact, had the least activity of all adult tissues (see Table 1). These results suggest that phase II O-acetyltransferase activation enzymes have decreased in relative amounts during the maturation of cardiac tissue.

Table 1
Tissue comparison of PhIP-DNA adduct levels obtained with N-OH-PhIP and cytosolic enzymes from newborn and adult F334 rats

Rat Tissue	RAL[#] × 10^{-6}/mg protein/30 min		
	O-Acetyltransferase	Sulfotransferase	Kinase
Newborn			
Heart	40.2 ± 7.8	ND*	0.32 ± 0.02
Kidney	27.0 ± 3.3	ND	0.26 ± 0.26
Lung	22.9 ± 5.1	0.10 ± 0.04	0.20 ± 0.02
Liver	21.0 ± 8.9	0.63 ± 0.40	1.96 ± 0.36
Muscle	11.3 ± 1.7	0.62[a]	0.53 ± 0.03
Brain	6.8 ± 2.9	ND	0.45 ± 0.15
Spleen	4.9 ± 0.7	0.06 ± 0.02	0.23 ± 0.11
Adult			
Liver	64.1 ± 21.0	0.06 ± 0.01	0.48 ± 0.10
Muscle	33.0 ± 6.6	0.17 ± 0.12	0.02 ± 0.01
Spleen	31.3 ± 1.9	0.04 ± 0.00	1.52 ± 0.60
Kidney	27.2 ± 0.8	0.08 ± 0.07	0.20 ± 0.03
Lung	20.1 ± 5.6	0.14 ± 0.04	0.13 ± 0.05
Brain	16.2 ± 3.0	0.19 ± 0.02	2.77 ± 0.74
Heart	14.0 ± 3.1	0.25[a]	0.34 ± 0.18

[#] RAL, relative adduct labeling is the number of PhIP-DNA adducts per million nucleotides of DNA.

* ND = Not Detected (RAL × 10^{-6}/mg protein/30 min < 0.01).

[a] Only one sample above background was observed. Adducts were isolated and quantified as described in Methods and materials. Cytosols were obtained from the pooled tissues of 16 newborn rats and 2 adult female rats as described in Methods and materials.

In keeping with the observation that PhIP does not cause liver tumors in rats [3,19,20], liver has been shown to be a tissue with low levels of PhIP-induced DNA damage in vivo [13,14,16,18]. Our observation that liver tissue of young adult female rats had very high O-acetyltransferase activity with N-OH-PhIP as a substrate was somewhat surprising although others have previously reported that rat hepatic O-acetyltransferase produced significant N-OH-PhIP binding to DNA [32,33]. Liver tissue in rats however has been shown to deal with dietary heterocyclic amines by several protective mechanisms [27,42]. Detoxification systems that are either constitutive or inducible may dominate metabolic processing of PhIP in liver tissue. For example, Kaderlik et al. [43] provided evidence that glutathione and N-glucuronidation of N-OH-PhIP played an important role in protecting rat liver DNA against PhIP-DNA adduct formation. Metabolic conjugation pathways involving glutathione and glucuronidation may explain in part why the induction of

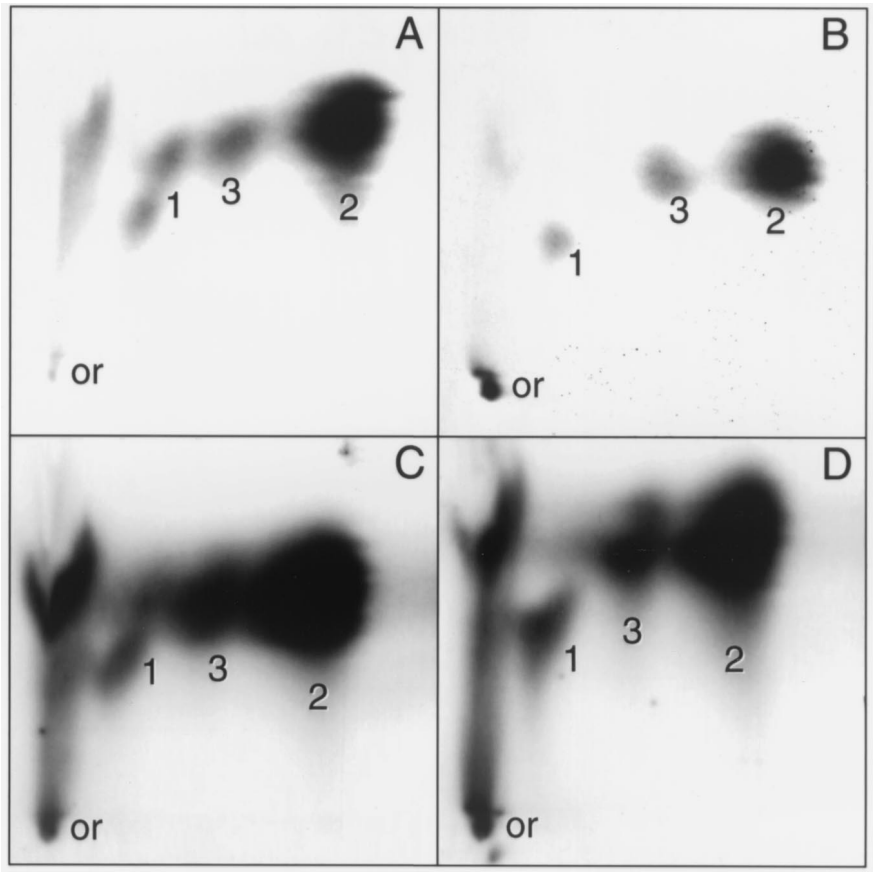

Fig. 4. PhIP-DNA adducts produced by O-acetyltransferases (A and C) or kinases (B and D). Reactions and chromatography were the same as described in the legend to Figure 1, except for kinase reactions 1 mM ATP replaced AcCoA. In Figure 4-A and 4-B, the cytosolic enzymes were from primary cultures of neonatal cardiac myocytes and in Figure 4-C and 4-D, the cytosolic proteins were from primary cultures of cardiac non-myocytes.

PhIP-DNA adducts is low in hepatocytes of PhIP-treated rats, and why liver is not a target organ for PhIP carcinogenesis in rats.

This is not the first study to examine phase II mutagen activation enzymes from rat tissues. Lin et al. [33] compared species differences in the biotransformation of N-OH-PhIP by hepatic cytosols from humans, rats, and mice, and found that O-acetyltransferase activity for rats cytosols was similar to that for human rapid aceylators but was about 10-fold higher than in mice. Rat sulfotransferases displayed the lowest activation of N-OH-PhIP among the three species, and ATP-dependent biotransformation of the mutagen by rat kinases was higher than human but lower than mouse kinases. An activation hierarchy of kinases > O-acetyltransferases > sulfotransferases was therefore determined for rat liver tissue [33]. Although we observed a similar disparity between cytosolic acetylation and sulfation, Lin et al. [33] detected substantially more kinase activation than measured in the present work. However, another investigation of in vitro phase II activation of N-OH-PhIP that used liver tissue from adult male F344 rats [32] determined esterification activities that agree well with the ones shown in Table 1 for liver. These latter studies included results that were interpreted as aminoacyl-tRNA synthetase activation of N-OH-PhIP [32]. The synthetase reaction contained both the amino acid L-proline and ATP. Covalent DNA binding of N-OH-PhIP showed a close correspondence between the L-proyl-tRNA synthetase reaction and an ATP-dependent activity, which the authors termed a “phosphatase” [32]. We did not detect any activation above background for L-proline-dependent reactions. In addition, PhIP-DNA adducts produced in the presence of L-proline and ATP did not exceed those formed by rat liver cytosolic proteins with ATP alone (data not shown). Lin et al. [33] previously noted that human and rodent cytosol-catalyzed DNA binding of N-OH-PhIP was not stimulated by the addition of L-proline. Therefore, these data suggest that L-proyl-tRNA synthetase does not participate in phase II activation of N-OH-PhIP.

One aim of this study was to learn more about mutagen metabolism in heart tissue because little is known about the effects of dietary mutagens in postmitotic mammalian cells and there is suggestive evidence that cooked meat mutagens can affect long-term cardiac function at the level of DNA [7,8,25,47]. Mechanisms by which dietary mutagen might induce deleterious changes in cardiac cell function have been considered by Gaubatz [25] and others [23,53]. The possibility that PhIP-DNA adducts may be linked to other effects other than tumorigenesis is suggested by results with

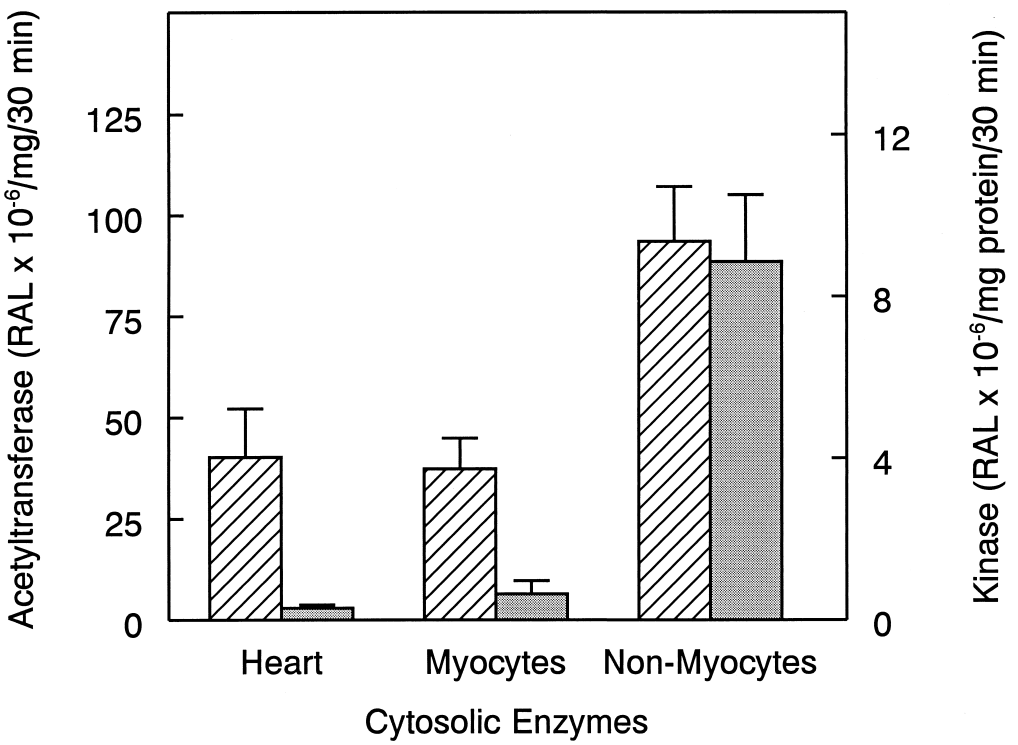

Fig. 5. Phase II enzyme activity levels comparing heart tissue and cultured cardiac cells. Cytosolic proteins were isolated from newborn rat heart tissue and cultured neonatal rat heart myocytes or non-myocytes and were assayed for kinase and O-acetyltransferase activation of N-OH-PhIP. ^{32}P-Postlabeling data are expressed as total adducts formed per protein in a 30 min reaction. Hatched bars represent O-acetyltransferase activity, and gray bars are kinase activity. Enzyme activities measured with non-myocyte cytosol were significantly different ($p < 0.05$) from activities obtained with heart and cardiac myocyte cytosols which were essential the same.

PhIP-treated animals that showed myocardial abnormalities, including areas of chronic inflammation and myocyte necrosis [23,48]. Based on these observations, heterocyclic amine-related DNA damage in heart might be causally related to pathological changes in this organ. Genomic damage from heterocyclic amines may in fact begin quite early. Davis et al. [49] showed that when rat pups were exposed to PhIP through the milk of lactating F344 mothers previously given an oral dose of the parent compound, PhIP-DNA adducts were induced in pup tissues. Five-day-old rats appeared to both detoxify and activate PhIP –the major form of the heterocyclic amine found in milk, and neonatal rat urine contained several PhIP metabolites, including N-OH-PhIP [49]. Therefore, exposure of newborns to dietary heterocyclic amines via breast milk may be relevant to cellular pathology if such exposures result in mutations to critical genes. Furthermore, some neonatal tissues, such as heart, may be targeted for more genomic damage due to their indigenous combination of phase II enzymes.

In contrast to the mutagen activation potential of newborn rat heart, adult heart tissue seemed to have a limited capacity to catalyze covalent DNA binding which would imply that myocardium is at less risk for genomic damage than other adult rat tissues. However, steady-state levels of DNA damage are a function of both damage induction and lesion removal, and studies have shown that the levels of PhIP associated with DNA decline with time, indicating that the association is either unstable or the damage is repairable [15]. The major DNA adduct formed with reactive derivatives of PhIP is dG-C8-PhIP [50]. While DNA repair of the bulky dG-C8-PhIP adduct has not been investigated in heart tissue, results that showed an accumulation of both alkyl- and aromatic-DNA adducts in postmitotic heart tissue of rodents during aging are consistent with inefficient or incomplete DNA repair in cardiac cells [51,52]. The fact that myocardium consists mostly of cells that are terminally differentiated and non-renewing probably contributes to adduct persistence in this tissue [44]. Thus, it is possible that an age-related appearance of some coronary diseases might stem from the accumulated effects of dietary mutagen exposures [25,44].

Since heart tissue is a mixture of cell types, it was important to establish whether the metabolism of N-OH-PhIP observed for the total organ was representative of cardiac myocytes or other cell types. Therefore, we cultured rat neonatal heart myocytes, which are a widely used model system to study the biochemistry and molecular biology of heart muscle [23,41]. For comparison, non-myocytes obtained from the same organs were put into primary culture. This latter culture was a heterogenous group of cells, composed predominately of fibroblasts. Enzyme activities obtained from primary cultures of neonatal cardiac myocytes

were essentially the same as those found for newborn heart thereby reflecting the tissue of origin, whereas proteins from fibroblast cultures had significantly different levels of acetyltransferase and kinase activity (Figure 5). These results support the use of cultured neonatal heart myocytes as an appropriate model to study the metabolism and toxicity of PhIP and other heterocyclic amines.

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