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Hamster exhibits major differences in organ-specific metabolism of the esophageal carcinogen N-nitrosodiethylamine

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ABSTRACT

Nitrosamines are carcinogens that require metabolic activation by CYP enzymes in order to exert their carcinogenic effect. Species differences exist in their esophageal carcinogenic potency, with the rat being the most sensitive and the Syrian hamster a resistant species. In the latter, the liver is the main target organ. This difference does not apply to directly acting N-nitroso compounds, suggesting that tissue-specific metabolic activation is involved in hamster esophageal resistance to nitrosamines. We have previously shown that Cytochrome P450 2A3 (CYP2A3) is responsible for N-nitrosodiethylamine activation in the rat esophagus. In order to find a mechanistic explanation for the resistance of hamster esophagus for nitrosamines, we have compared the metabolism of NDEA between esophagus and liver of the hamster.

Hamster esophagus is capable of activating NDEA ($K_m = 1.02 \pm 0.44\ \mu M$ and $V_{max} = 1.96 \pm 0.26$ nmol acetaldehyde/min/mg microsomal protein). However, the hamster liver showed a 40-fold higher catalytic efficiency (V_{max}/K_m) towards NDEA metabolism compared with its esophagus. Hamster esophagus expresses CYP2A8, CYP2A9 and CYP2A16, but not CYP2E1. An antibody against human CYP2A6 was able to inhibit NDEA metabolism in hamster esophageal, but not liver microsomes.

Our results suggest that in the hamster esophagus, but not in the liver, most of the NDEA is metabolized by CYP2A enzymes, but with a rather poor efficiency when compared to the liver. This is in accordance with previous results showing that for the hamster, the main target organ of NDEA is the liver.

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1. Introduction

N-nitroso compounds form a large class of carcinogens, and are the only potent chemicals known to induce esophageal tumors in experimental animals. Considerable evidences suggest that some of these carcinogens found in tobacco-related products are also involved in the induction of esophageal tumors in humans (Hecht, 2003; Wogan et al., 2004). Some of the N-nitroso compounds, like N-nitrosamides, are directly acting: they decompose readily at physiological pH to alkylating agents, which can give rise to mutations and initiate tumors (Schoental, 1961; Montesano and Hall, 1984; Dietrich et al., 2005). Since these compounds do not require metabolic activation in order to exert their carcinogenic effects, the sites of tumor formation are restricted to the sites of exposure. The second group consists of N-nitrosamines, which are stable at physiological pH and in aqueous solutions (Magee and Schoental, 1964), and require metabolic activation. The efficiency of nitrosamine metabolism, a consequence of the amounts and catalytic properties of nitrosamines metabolizing CYP enzymes in different tissues, is thought to be fundamental for their tissue-specific carcinogenicity (Liteplo and Meek, 2001).

Many nitrosamines induce esophageal tumors and for some of them the esophagus is the only target organ (Craddock, 1993). However, the esophageal susceptibility to nitrosamines varies greatly among experimental animals: rat is the most susceptible species, whereas Syrian hamster is resistant. According to Lijinsky (1992), more than 50% of all nitrosamines tested induce esophageal tumors in rats. By contrast, for hamsters the main target organ of nitrosamines is the liver, where up to 60% of the compounds tested induced tumors. None of the nitrosamines is known to induce tumors in the hamster esophagus. Instead, directly acting alkylating agents caused esophageal tumors both in rats and hamsters (Schoental and Magee, 1962; Schoental, 1963; Magee and Schoental, 1964; Herrold, 1966; Lijinsky, 1992). For instance, intragastric administration of N-nitroso-N-methylurethane (NMUR) to hamsters led to the development of esophageal tumors in more than 80% of the animals (Herrold, 1966).

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These results suggest that tissue susceptibility for nitrosamines must depend, among other factors, on differential metabolic activation in the liver and the esophagus. To our knowledge no comparative studies have been carried out on the hepatic and esophageal metabolism of nitrosamines in hamster. Such a study could help, however, to understand the mechanism behind tissue susceptibility and resistance to nitrosamines.

We have previously shown that CYP2A3 is expressed in the rat esophagus, but not liver, and is mainly responsible for the activation of N-nitrosodiethylamine (NDEA) in the former (Ribeiro Pinto et al., 2001). No data is available neither on CYP2A expression nor on its possible role in nitrosamine metabolism in the esophagus of hamsters.

In an attempt to explain differences regarding tissue specificity towards nitrosamine-induced tumors, we in the present study characterized CYP2A expression in the esophagus of Syrian hamsters and carried out a comparative kinetic analysis on the hepatic and esophageal metabolism of NDEA. Furthermore, we wanted to test whether or not differences in the metabolic profile of NDEA is the underlying mechanism between the different susceptibility of rat and hamster to NDEA carcinogenesis. It appears that whereas the hamster esophagus expresses CYP2A enzymes that are involved in the metabolism of NDEA, its hepatic metabolism, which is not carried out by CYP2A enzymes, is extremely efficient by comparison. It appears therefore that, due to the extremely efficient hepatic NDEA metabolism, its metabolic activation in hamsters occurs preferably in liver in detriment of esophagus, thus giving rise mainly to hepatic tumors.

2. Materials and methods

2.1. Animals

Male Syrian Golden Hamsters (8–9 weeks, 100 ± 10 g) provided by the Laboratory of Experimental Surgery, UERJ, were kept in 12-h light and dark cycle and were given food and water *ad libitum*. The animals were starved overnight and killed by CO_2 asphyxiation. The liver and esophageal mucosa were quickly removed to ice-cold homogenizing buffer (50 mM Tris pH 7.4/1.15% KCl) and one aliquot of each tissue was immediately placed on TRIZOL® for subsequent RNA extraction. The remaining samples were frozen at −70 °C for microsomal preparation.

2.2. RT-PCR

Total RNA was extracted from the esophageal mucosa and liver using the TRIZOL® reagent following the manufacturer's instructions (Invitrogen, SP, Brazil). Samples were then treated with DNase RQ1 RNase Free (Promega, SP, Brazil) according to manufacturer's instructions to avoid any contaminating DNA. The RNA was then quantified by spectrophotometry and 2 μg of RNA were used for reverse transcription using MMLV-RT (Invitrogen, SP, Brazil), as previously described (Robottom Ferreira et al., 2003). The PCR reactions were done using 1 μL of the RT reaction mixture and specific oligonucleotide primers for CYP2A8, CYP2A9 and CYP2A16. The oligonucleotide sequences for the amplification of CYP2A9 are described elsewhere (Kurose et al., 1998). The oligonucleotide primers for the amplification of CYP2A8 and CYP2A16 were designed by us by aligning the two mRNAs sequences using the program ClustalX, version 2.0 for Windows (Thompson et al., 1997) and selecting the primers within the regions that were unique for each isoform. Oligonucleotide sequences and PCR conditions used are shown in Table 1. Ten microliters of the PCR mixture was separated on 6% polyacrylamide gel and silver stained (Sanguinetti et al., 1994).

Table 2
ECOD, COH and NDEAd activities of hamster esophageal and hepatic microsomes.

	Esophageal	Hepatic
ECOD	16.74 ± 3.75	934.22 ± 73.85
COH	3.98 ± 0.59	77.04 ± 5.88
NDEAd	1.65 ± 0.25	3.51 ± 0.01

Results are expressed as mean ± S.D. of three determinations, and given as pmol umbelliferone/min/mg microsomal protein for ECOD and COH and nmol acetaldehyde/min/mg microsomal protein for NDEAd.

2.3. Microsomal preparation and Western blotting

Esophageal and liver microsomes were prepared as previously described (Ribeiro Pinto et al., 2001). Protein concentration was determined by the method of Lowry et al. (1951). For Western blotting analysis, samples of esophageal or liver microsomal proteins (50 μg or 25 μg, respectively) were electrophoresed in an 8% resolving gel. After electrophoresis the gel was electroblotted to a nitrocellulose membrane (BioRad, SP, Brazil). The membrane was then blocked and incubated either with an anti-mouse CYP2a5 (Lang et al., 1989) or an anti-CYP2E1 (Millipore, MA, USA) primary antibody diluted 500-fold followed by the incubation with the secondary antibody (Anti-rabbit IgG conjugated to Alkaline Phosphatase, Sigma, SP, Brazil) diluted 1:10,000. Detection was performed with the Alkaline Phosphatase kit according to the manufacturer's instructions (BioRad, SP, Brazil).

2.4. Measurement of enzymatic activities

Coumarin 7-hydroxylation (COH) activity of liver microsomes was measured as described by Aitio (1978) by incubating 0.1 mg of microsomal protein with 100 μM coumarin and 360 μM NADPH for 5 min in a total reaction volume of 500 μL. COH activity of esophageal microsomes was determined as for the liver, except that the reaction was carried out for 30 min. 7-Ethoxycoumarin-*O*-deethylase (ECOD) activity catalyzed by liver and esophageal microsomes was carried out as for COH, except that the substrate was ethoxycoumarin (300 μM), the protein amount was 0.05 mg (liver) or 0.1 mg (esophagus) and the incubation times were 5 and 30 min for liver and esophageal microsomes, respectively. Protein concentration and incubation time were tested and product formation was linear under the conditions described above. N-nitrosodiethylamine deethylation (NDEAd) activity was determined as described previously (Ribeiro Pinto et al., 2001) by incubating 25 μg of microsomal protein with 100 μM NDEA and 720 μM NADPH for 5 min (liver microsomes) or 10 min (esophageal microsomes) in a total reaction volume of 100 μL. For kinetic constant determinations of NDEA metabolism by hepatic and esophageal microsomes, the reaction had varying NDEA concentrations. Data analysis was done using the GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego CA, USA). To investigate whether an inhibitory polyclonal antibody against human CYP2A6 (Xenotech LLC, KS, USA) could inhibit the microsomal NDEAd activities, variable amounts were incubated with liver or esophageal microsomes for 5 min at 37 °C prior to the addition of the substrate.

3. Results

The metabolism of ethoxycoumarin, coumarin and NDEA by esophageal and hepatic microsomes was initially investigated. Ethoxycoumarin is a good substrate of CYP2A enzymes (Honkakoski et al., 1993) and coumarin is a specific substrate for some CYP2A enzymes such as CYP2A3, CYP2a5, CYP2A8 and CYP2A16, but is not metabolized by hamster CYP2A9 (Honkakoski et al., 1988; Raunio et al., 1988; Pelkonen et al., 1994a; Kurose et al., 1998). NDEA is known to be a substrate of both CYP2E1 and CYP2A (Yoo et al., 1990; Camus et al., 1993; Ribeiro Pinto et al., 2001). The enzymatic activities are shown in Table 2. It appears that whereas ECOD and COH

Table 1
PCR conditions: oligonucleotide primer set, annealing temperature, cycle number and amplicon size.

Gene	Oligonucleotide primers	Annealing temperature (°C)	Cycle	Fragment size (bp)	Ref.
CYP2A8	5′-AGCCTGGCTAAGATGGAG-3′ (sense) 5′-AACCCGTCTTCAGGAAGG-3′ (antisense)	56	40	341	See Section 2
CYP2A9	5′-CATTCCTCGGAAGAAGACTAT-3′ (sense) 5′-CCACCTTGGTTGGTTCC-3′ (antisense)	54	40	399	Kurose et al. (1998)
CYP2A16	5′-TGAAGTGTTTCCTATGCTGG-3′ (sense) 5′-CTCATCCAGCCCTGGATC-3′ (antisense)	58	40	334	See Section 2

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