Nitroreduction and Formation of Hemoglobin Adducts in Rats with a Human Intestinal Microflora

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In the covalent binding of nitroarenes to macromolecules, nitroreduction is an important step. The intestinal microflora represents an enormous potential of bacterial nitroreductase activity. As a consequence, the *in vivo* nitroreduction of orally administered nitroarenes is primarily located in the intestine. In this study, we have investigated the nitroreduction of 2-nitrofluorene (2-NF) by a human microflora in female Wistar rats. Germ-free (GF) rats were equipped with a bacterial flora derived from human feces. Nontreated GF rats and GF animals equipped with a conventional rat flora were used as controls. The composition of the human and the conventional microflora isolated from the rats were consistent with the microflora of the administered feces. In the rats receiving only sunflower seed oil, no adducts were detected. The animals equipped with a human or rat microflora that received 2-aminofluorene (2-AF) formed 2-AF hemoglobin (Hb)-adducts at average levels (mean ± SEM) of 5.3 ± 0.3 and 6.7 ± 0.7 μmole/g Hb, respectively. After 2-NF administration, the adduct levels were 0.022 ± 0.003 and 0.043 ± 0.010 μmole/g Hb, respectively. In the GF rats, an adduct level of 0.57 ± 0.09 was determined after 2-AF administration and no adducts were detected after 2-NF administration. The results show that nitroreduction by an acquired human intestinal microflora and subsequent adduct formation can be studied in the rat *in vivo*. — Environ Health Perspect 102(Suppl 6):39–41 (1994)

Key words: nitro-PAH, nitroreduction, 2-nitrofluorene, human microflora, hemoglobin adducts, germ-free rats

Introduction

In the covalent binding of nitroarenes to macromolecules, nitroreduction is the first important step in *N*-directed metabolic activation (*1*). The nitro group can either be partially reduced to form a nitroso or an *N*-hydroxyl intermediate or be completely reduced to an amine (*2,3*). The amine can be reoxidized to form *N*-hydroxyl products and be further oxidized in the erythrocyte to form a nitroso compound. This is a reactive species that can bind covalently to hemoglobin (Hb) (*4,5*).

Unlike other routes of biotransformation, nitroreduction is not performed by the intrinsic pool of enzymes present in body tissues but primarily by enzymes of bacteria present in the intestine (*6,7*). *In vivo* only a minor contribution is supplied by liver enzymes (*8,9*) or, presumably, by enzymes in the lung (*10,11*). The intestinal microflora represents an enormous potential of bacterial nitroreductase activity. Its metabolic capacity depends on the presence of a community of hundreds of aerobic and anaerobic bacterial species representing several billion individual microorganisms. Each of the bacterial strains can be considered an autonomic metabolic unity. In this study, we have investigated the formation of Hb-adducts in germ-free (GF) rats that were equipped with a human intestinal microflora and in GF animals that were supplied with a conventional rat microflora. We have tried to establish the importance of bacterial nitroreduction as a first conditional step in a cascade of metabolic activation reactions eventually leading to macromolecular binding such as Hb adduct formation.

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Methods

Animals

Home-bred GF female Wistar rats (Cpb:WU) were equipped with either a bacterial flora derived from human feces or a rat flora. Rats without a microflora were used as controls. The animals weighed 130 to 210 g, were housed individually in plastic isolators, and had free access to autoclaved sterilizable rat and mouse diet and water.

Microbiology

One and two weeks before the administration of the aryl compounds the microflora was given by gavage. After collection of the blood, the cecum was carefully tied up and weighed. In the content of the cecum, the total vital bacteria count was determined. In addition, the following bacterial genera were characterized by standard methods (*12*) and counted: *Enterobacteriaceae*, *Staphylococci*, *Streptococci* (aerobically cultured), and *Lactobacilli*, *Clostridia*, *Bacteroides*, *Bifido bacteria*, and *Veillonella* (anaerobically cultured). The abundance of yeasts and fungi was also determined.

Administration

In each of the groups of GF rats equipped with a microflora, three rats received orally 1 mmole/kg 2-nitrofluorene (2-NF) dissolved in sunflower seed oil (SSO), three rats received 1 mmole/kg 2-aminofluorene (2-AF) in SSO and three rats were given only SSO. Two GF rats received 2-AF in SSO, two rats received 2-NF in SSO, and two were given SSO only.

Chemicals

2-Aminofluorene (98%) was supplied by Aldrich Europe (Bornem, Belgium). 7-Fluoro-2-nitrofluorene (>99.8%) was obtained from Sigma (St. Louis, MO).

Table 1. Microbiologic characterization of rat and human feces and the microbiologic status (in logarithmic bacterial counts) of GF Wistar rats after 2 weeks of incubation with rat- and human-derived microfloras.

	Rat microflora		Human microflora	
	Cecum	Feces	Cecum	Feces
Aerobic				
Total aerobes	8.3 ± 0.2	9.5	8.9 ± 0.5	9.2
Streptococcus sp.	6.5 ± 0.6	9.4	8.4 ± 0.4	6.5
Staphylococcus sp.	4.1 ± 0.9	4.3	1.8 ± 0.1	6.3
Yeasts and fungi	<2.7 ± 0.1	<2.9	1.8 ± 0.1	3.5
Anaerobic				
Total anaerobes	10.1 ± 0.2	9.8	10.5 ± 0.3	10.3
Enterobacteriaceae	7.2 ± 0.9	5.7	8.6 ± 0.5	9.0
Lactobacillus sp.	7.7 ± 0.3	8.2	8.3 ± 0.7	8.4
Bacteroides sp.	9.2 ± 0.4	8.8	10.1 ± 0.3	9.4
Clostridium sp.	8.6 ± 0.5	9.2	9.3 ± 0.3	9.0
Bifidobacterium sp.[a]	<2.8 ± 0.1	<2.9	8.1 ± 0.4	8.8
Veillonella sp.	<3.0 ± 0.1	<2.9	4.6 ± 0.4	7.1
Total count	10.8 ± 0.2	11.4	11.1 ± 0.1	11.1

[a] *B. fragilis* group.

Heptafluorobutyric anhydride (HFBA) and 2-nitrofluorene (98%) were supplied by Janssen Chimica (Geel, Belgium), isooctane (HPLC-grade) from Fluka Chemica (Brussels, Belgium), and SSO from OPG (Utrecht, The Netherlands). Amberlite XAD_2 was supplied by Serva (Heidelberg, Germany). 2-Amino-7-fluorofluorene was obtained by the reduction of the aforementioned 7-fluoro-2-nitrofluorene with hydrazine-monohydrate and Raney Nickel. All other solvents used were HPLC-grade (Lab-Scan Analytical Sciences, Dublin, Ireland).

Blood Analysis

Forty-eight hours after the administration of the aryl compounds, blood was collected by heart punction. After isolation of the Hb, unbound 2-AF was removed by solid phase extraction using XAD_2 (*13*). The Hb concentration was determined according to the hemiglobincyanide method (*14*). The adducts were subsequently hydrolyzed under mildly basic conditions (*15*). 2-Amino-7-fluorofluorene was added to serve as an internal standard. The solution was extracted overnight with XAD_2. After removing the Hb hydrolysate, the solid sorbent was washed and dried. The arylamines were eluted with toluene and derivatized with HFBA. 2-AF was determined using GC–MS (coefficient of variance = 14.1%).

Results

The characterization of the microbiologic status of the rats is presented in Table 1. The intestines of the previously GF rats were efficiently colonized by both aerobic and anaerobic species. The integrity of the microflora is preserved considering the colonization of all genera of bacterial species that were identified. There are only small differences between the cecum populations among the animals receiving a rat and a human microflora.

The counts of *Streptococcus* are higher in the administered rat feces than in the ceca of the GF rats receiving this microflora. The genera *Enterobacteriaceae* and *Bacteroides* are more abundant relative to the microflora of the conventional feces. In the ceca of rats receiving the human microflora *Staphylococcus*, *Veillonella*, yeasts, and fungi are less abundant as compared to the administered feces. Aerobic as well as anaerobic culturing and gram preparations of the cecum content indicated that the ceca of the GF animals contained no microflora.

In Table 2 the relative cecum weights are presented. The ceca of the GF rats had a four times higher relative weight as compared to the ceca weights of the animals that received either a human or a rat microflora. There is a minor difference between the animals that received a microflora derived from human feces and the animals receiving a rat feces-derived flora. This difference is significant ($p<0.05$) in the two-tailed t-test.

Table 3 presents the Hb adduct levels that were determined in the blood of nontreated GF rats, GF animals equipped with a human microflora, and GF animals with a conventional rat microflora. The relative adduct levels (± SEM) determined in the blood of rats that received 2-NF, as compared to the adduct levels observed in animals receiving 2-AF amounting to 0.3 ± 0.1% and 0.8 ± 0.2% in animals equipped with a human and a conventional rat microflora, respectively.

Discussion

The composition of the human and the conventional microflora isolated from the rats was consistent with the microflora of the source material (feces). The difference in Hb-adduct formation of 2-NF between animals equipped with a human-derived microflora and animals equipped with a flora originating from rat feces is a factor also. This difference is not statistically significant. Regarding the results obtained in the GF rats, the nitroreducing capacity is mainly located in the microflora. Differences in the amounts of formed Hb adducts could be related to the nitroreducing capacity of the microflora. It is not known what bacterial species or yeasts and fungi are involved in this metabolic step. From *in vitro* experiments it is known that some anaerobic species such as *Peptostreptococcus* and *Peptococcus* species, *Bacteroides thetaiotaomicron*, *Clostridium perfringens*, and *Clostridium* species are capable of nitroreduction of 1-nitropyrene (*16*) and 6-nitrobenzo[*a*]pyrene (*17*). Aerobic species have also been shown to reduce nitro aromatics to some extent (*18,19*). The rat and human intestinal microflora were found to be capable of almost complete conversion of 6-nitro-benzo[*a*]pyrene (*16*) and 1-nitropyrene (*6*). Human intestinal microflora is known to reduce aromatic nitro compounds *in vitro* (*6,16*).

Table 2. Relative cecum weights (± SEM) of GF rats and rats that received a feces-derived rat microflora and a human microflora, respectively.

	No microflora ($n=6$)	Rat microflora ($n=9$)	Human microflora ($n=9$)
Relative cecum weights, %	7.6 ± 0.4	2.1 ± 0.1	1.5 ± 0.1

Table 3. Levels of 2-AF Hb adducts (μmole/g Hb ± SEM) in rats that received either 1 mmole/kg 2-nitrofluorene or 1 mmole/kg 2-aminofluorene by gavage.

Compound	No microflora	Rat microflora	Human microflora
2-nitrofluorene	ND[a]	0.043 ± 0.010	0.022 ± 0.003
2-aminofluorene	0.57 ± 0.09	5.3 ± 0.3	6.7 ± 0.7

[a] Not detected at a detection limit of approximately 0.1 pmole per injection or 0.001 μmole 2-AF/g Hb.

When the levels of Hb adducts determined in the blood of animals that received a dose of 2-AF were set on 100%, the levels observed in animals receiving 2-NF at the same dose were found to be 0.8% in animals equipped with a conventional rat microflora. This represents the contribution of the nitroreducing metabolic pathway to covalent binding with Hb. Calculations on the results, previously obtained by Suzuki and coworkers (*2*) in Sprague-Dawley rats that received 0.5 mmole/kg by gavage gave a value of 0.9%. The extent of binding to Hb is probably limited by *N*-hydroxylation (*2*) or *O*-acetylation (*20*). The difference in adduct formation cannot be explained by any of the bacterial groups presented in Table 1. Because it is known that a wide range of intestinal bacterial species are able to reduce aromatic nitro compounds (*16*), it is possible that species other than the ones that were identified from the cecum contents are responsible for the observed difference in adduct formation. Qualitative differences in the nitroreducing potential between the microflora from humans, rhesus monkeys, and rats are reported (*21*). The difference between the Hb adduct levels formed by the control animals equipped with a human or rat microflora are not statistically significant. In the GF rats, no 2-AF Hb adducts could be detected after 2-NF administration. The GF rats receiving 2-AF formed adducts at a much lower level compared to the animals with a feces-derived microflora. We suggest that this difference is due to the GF status of the rats and may be related to a change in the resorption of 2-AF from the intestine.

This study shows that nitroreduction by an acquired human intestinal microflora and subsequent adduct formation can be studied in the rat *in vivo*. The intestinal microflora contains a metabolic system principally responsible for *in vivo* nitroreduction. This turns out to be a critical step in the Hb adduct formation. We did not observe a statistically significant difference between the adduct formation in rats equipped with a human microflora or a conventional rat microflora. Because of the maternal origin of the microflora in humans, interindividual differences in the composition can be expected. However, in this study we did not consider possible differences in nitroreducing capacity of the microflora of different individuals. The distribution pattern of nitroreducing capacities of human microfloras is an interesting topic in the human risk evaluation of exposure to mixtures of nitro aromatics such as diesel exhaust emissions.

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