Metabonomic Identification of Two Distinct Phenotypes in Sprague-Dawley (Crl:CD(SD)) Rats

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Genetic drift in animal populations has been a recognized concern for many years. Less understood is the potential for phenotypic “drift” or variation that is not related to any genetic change. Recently, stock Sprague-Dawley (Crl:CD(SD)) rats obtained from the Charles River Raleigh facility demonstrated a distinct endogenous urinary metabonomic profile that differed from historical control SD urine spectral profiles obtained over the past several years in our laboratory. In follow-up studies, the origin of the variant phenotype was narrowed down to animals of both sexes that were housed in one specific room (Room 9) in the Raleigh facility. It is likely that the two phenotypes are related to distinct populations of gut flora that particularly impact the metabolism of aromatic molecules. The most pronounced difference between the two phenotypes is the relative amounts of hippuric acid versus other aromatic acid metabolites of chlorogenic acid. Though both molecular species are present in either phenotype, the marked variation in levels of these molecules between the two phenotypes has led to the designation of high hippuric acid (HIP) and high chlorogenic acid metabolites (CA) phenotypes. Specific urinary components that distinguish the phenotypes have been thoroughly characterized by NMR spectroscopy with additional, limited characterization by LC-MS (high performance liquid chromatography coupled with mass spectrometry). Co-habitation of rats from the two phenotypes rapidly facilitated a switch of the CA phenotype to the historical Sprague-Dawley phenotype (HIP). The impact of these variant phenotypes on drug metabolism and long-term safety assessment studies (e.g., carcinogenicity bioassays) is unknown.

Key Words: metabonomics; phenotype; Sprague-Dawley (Crl:CD (SD)); hippuric acid; chlorogenic acid.

Metabonomics is a powerful tool that assesses the biological response to stress, such as that induced by toxins or disease states. However, any physiological or environmental change, such as age, estrus, diet, and housing can impact an animal’s metabonomic profile (Beckwith-Hall et al., 2002a,b; Bollard et al., 2001; Phipps et al., 1998) have noted instances where this has not been the case in urines collected from several strains. In these instances, unusually low or undetectable hippuric acid levels were observed in urine from these rat strains with concomitant increases in other urinary aromatic species including 3-(3-hydroxyphenyl)propionic acid (3-HPPA) and 3-hydroxycinnamic acid (3-HCA). In some cases, the dietary intake was implicated in the observed change in hippuric acid excretion. In other cases, the cause was more difficult to ascertain. Since gut flora are known to be involved in the metabolism of plant phenolics, it has been postulated that changes in the population of gut flora are also involved in instances where low levels of hippuric acid are excreted in the urine (Gavaghan et al., 2001).

In the course of five years of metabolomics studies conducted in this laboratory, employing mainly Wistar and Sprague-Dawley rats, the urinary metabolite profile within both strains has remained remarkably stable with high levels of hippuric acid as a prominent hallmark metabolite. In this report, we characterize a recent change in that stability with two distinct phenotypes coming from different rooms (colonies) in the same commercial facility.

MATERIALS AND METHODS

Animals. Sprague-Dawley (Crl:CD(SD)) rats were obtained from Charles River Laboratories. Animals from historical studies (Table 1) were obtained from the facilities listed. Rats for phenotype follow-up experiments were
obtained exclusively from the Charles River Raleigh facility. Room 9 at that facility was found to house animals with the variant phenotype and Room 10 housed animals with the historical phenotype observed in previous studies with SD rats from various Charles River facilities. Animals in historical studies ranged from 6 to 12 weeks of age, and all rats used for the phenotype assessment studies were between 7 and 8 weeks of age at initiation.

Routine animal husbandry. Animals were housed in an AAALAC accredited facility and all in vivo studies were reviewed and approved by the IACUC. When not in metabolism cages, animals were housed in individual cages in temperature (70–78°F) and humidity (30–70% RH) controlled rooms ad libitum. Food and water were available ad libitum. For phenotype monitoring, animals were received from Charles River and placed directly into metabolism cages without any acclimation. Food and water were available ad libitum. Urine collection.

When required, animals were placed into individual plastic metabolism cages (Harvard Apparatus, Holliston, MA) where they remained for the duration of urine collection. Food and water were available ad libitum. For the time course study, initial urine samples were obtained as described above with additional samples (3 × 24 h) collected on a weekly basis for four weeks (only a single 24 h sample was collected during week 4). Urine was collected into tubes containing 1 ml of 1% sodium azide and maintained at 0°C.

Cohabitation experiment. To assess a possible change in phenotype, a group of 10 female rats from Rooms 9 and 10 were obtained and baseline phenotype identified as described above. The rats were then pair housed (one from each room) in plastic shoebox cages containing Bed O’Cob absorbent bedding. Food and water were available ad libitum. At weekly intervals, individual animals were placed in metabolism cages for 24 h and a single urine sample was subsequently collected, after which each animal was then returned to its shoebox cage. Following three weeks of cohabitation, animals were separated into individual shoebox caging. Weekly 24-h urine collections were continued for an additional two weeks to explore the possibility of reverting back to the original phenotype.

One-dimensional 1H NMR spectroscopy. Samples for NMR analysis were prepared by mixing 500 μl of urine with 250 μl of buffer in 96-well plates. The buffer was added to provide some normalization of the urinary pH. After mixing, plates were centrifuged to sediment insolubles. The buffer was 0.2 M sodium phosphate buffer at pH 7.4 (80:20 H2O:D2O), containing 1 mM TSP (sodium 2,2′,3,3′-deutero-3-trimethylsilylpropionate, an internal NMR reference standard) and 3 mM sodium azide. 1H NMR data were acquired using a Varian Inova NMR spectrometer operating at 600.36 MHz for 1H and equipped with a 1H-[15N, 13C] flow probe (120 μl active volume) and a Varian automated sample transport accessory (VAST). Four-hundred-fifty microliters of sample was injected into the probe with no push solvent. Two cell rinses were completed with isotonic phosphate buffer between samples. The push buffer was prepared by mixing one part 0.2 M sodium phosphate buffer at pH 7.4 with two parts water. One-dimensional 1H NMR spectra were acquired at 27°C using a one-dimensional NOESY pulse sequence including water presaturation and a mixing time of 100 milliseconds. A total of 64 scans were collected with 64k data points, an acquisition time of 2.73 s, an inter-pulse delay of 1 s, and a sweep width of 12 kHz.

NMR data analysis. Spectra were processed and analyzed using in-house software, Metabonomi (International Patent Application, WO2004038602A1). Spectra were normalized to the total integrated spectral area minus the region containing water and urea from 6.0 to 4.5 ppm.

Mass spectrometry. Samples were centrifuged and injected directly onto the HPLC column undiluted for metabolite profiling and characterization. For
RESULTS

Phenotype Identification and Characterization:

Prominent differences between the phenotypes were noted in $^1$H NMR spectra. Based on the clear and consistent predominance of aromatic components in the urine, hippuric acid, or chlorogenic acid metabolites, these two phenotypes are referred to as HIP (high hippuric acid) or CA (high chlorogenic acid metabolites). A selected region of $^1$H NMR spectra of urine from SD rats of HIP and CA phenotypes is shown in Figure 1. After reviewing records of this laboratory’s metabonomic studies conducted using Charles River-supplied rats, the unique source of the CA phenotype was identified as the Raleigh facility, Room 9. A summary of metabonomic experiments is shown in Table 1 including the Charles River facility and room of origin and date of shipment receipt. In additional studies with Charles River-supplied rats from the Raleigh facility, the HIP phenotype was observed in male and female SD rats from Room 10, while the CA phenotype was observed in male and female SD rats from Room 9. Major metabolites contributing to each phenotype were identified and levels quantitated using NMR, LC-MS, and combined LC-NMR/MS methods. The HIP and CA metabolite profiles are detailed in Table 2. NMR spectral assignments were completed using a combination of literature (Gavaghan et al., 2001; Nicholls et al., 2003) and commercially available authentic standards in all cases except the dihydroquinolinone glucuronide associated with the HIP phenotype. Profiling experiments by LC-MS revealed that a metabolite at $[M+H]^+$ m/z 340 was abundant in the Room 10 sample, while present only at trace levels in the Room 9 sample. A representative subset of samples from male and female rats, originating from both rooms, was analyzed to confirm this finding. Both source-induced and collision-induced fragmentation yielded a fragment ion at m/z 164 amu consistent with a neutral loss of 176 amu, indicative of a glucuronide conjugate. Accurate mass values were determined to be m/z 340.1016 and m/z 164.0679 amu. Based on these values, the proposed formula for the unknown at m/z 340 is $C_{13}H_{18}NO_8$ and the fragment at m/z 164 is consistent with $C_9H_4NO_2$ (both < 5 ppm mass deviation). Further experiments using an ion trap mass spectrometer, on-line LC-NMR/MS experiments, as well as NMR experiments on the isolated material led to the proposed dihydro-quinolinone structure (Fig. 3), a cyclic derivative of cinnamic acid. The MS fragmentation pattern and $^1$H NMR spectrum are shown in Figures 4 and 5, respectively. Authentic 3,4-dihydro-2(1H)-quinolinone (98% pure, Sigma-Aldrich Company) fragmented in a manner similar to the aglycone, with the predominant product ion forming from loss of 42 amu. The proposed assignment was further validated through comparison of NMR spectral data with authentic 3,4-dihydro-2(1H)-quinolinone and reference to $^1$H chemical shift reference tables that quantify the effect of substituents on chemical shifts (Pretsch et al., 2000).
Stability of Phenotype

The metabolite profile of 10 rats from each phenotype was monitored in SD male rats for a period of four weeks. During this time, the rats were individually housed. After four weeks, the metabolite profile in the urine remained constant for 19 out of the 20 animals. One animal, initially having the CA phenotype, showed a different metabolite profile after the housing period.

**FIG. 1.** Region of $^1$H NMR spectra of urine from male SD rats, HIP and CA phenotypes. The region from 6.7 to 8.0 ppm is shown. Resonances for hippuric acid, phenylacetylglucine, and chlorogenic acid metabolites 3-(3-hydroxyphenyl)propionic acid (1) and 3-(4-hydroxyphenyl)propionic acid (2) are labeled on the spectra. The inset graph shows data from individual animals where the x-axis is hippuric acid peak area and the y-axis is the chlorogenic acid metabolites peak area. Data points from Room 9 rats are shown in squares while data from Room 10 rats are shown in circles. Urine from Room 9 rats consistently contains low hippuric acid levels and high levels of chlorogenic acid metabolites while urine from Room 10 rats consistently contains high hippuric acid levels and low levels of chlorogenic acid metabolites. Similar metabolic profiles are also observed in female rats from Room 9 and 10.

**TABLE 2**
Summary of Relative Abundance of Urinary Metabolites from HIP and CA Phenotypes in Male SD Rats

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>HIP</th>
<th>CA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hippuric acid</td>
<td>1.0</td>
<td>0.2</td>
</tr>
<tr>
<td>N-methylnicotinamide</td>
<td>1.0</td>
<td>1.8</td>
</tr>
<tr>
<td>3-(3-hydroxyphenyl)propionic acid</td>
<td>1.0</td>
<td>5.3</td>
</tr>
<tr>
<td>3-(4-hydroxyphenyl)propionic acid</td>
<td>1.0</td>
<td>13.0</td>
</tr>
<tr>
<td>Dihydro-quinoline glucuronide*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown 1 (group of aromatic resonances at 7.22 ppm)</td>
<td>1.0</td>
<td>2.6</td>
</tr>
<tr>
<td>Unknown 2 (singlet at 6.58 ppm)</td>
<td>1.0</td>
<td>2.9</td>
</tr>
<tr>
<td>Unknown 3 (singlet at 6.53 ppm)</td>
<td>1.0</td>
<td>8.6</td>
</tr>
<tr>
<td>Unknown 4 (doublet at 1.38 ppm)</td>
<td>1.0</td>
<td>6.5</td>
</tr>
<tr>
<td>Unknown 5 (multiplet at 1.18 ppm)</td>
<td>1.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Unknown 6 (multiple resonances in sugar region)</td>
<td>1.0</td>
<td>0.5</td>
</tr>
</tbody>
</table>

*Component detected by MS in HIP phenotype and present at only trace levels in CA phenotype. The difference is noted in the $^1$H NMR spectra but overlapping resonances make quantitation by NMR spectroscopy difficult.

**FIG. 2.** Urinary hippuric acid levels in female SD rats before and after cohabitation. Results show that urinary hippuric acid levels within the CA phenotype normalized to the levels associated with the HIP phenotype after cohabitation. One standard deviation is displayed on the graph.

**Stability of Phenotype**

The metabolite profile of 10 rats from each phenotype was monitored in SD male rats for a period of four weeks. During this time, the rats were individually housed. After four weeks, the metabolite profile in the urine remained constant for 19 out of the 20 animals. One animal, initially having the CA phenotype, showed a different metabolite profile after the housing period.
phenotype, spontaneously began excreting urine having a metabolite profile consistent with the HIP phenotype after two weeks. For the remaining two weeks of monitoring, the urine metabolite profile of that animal remained consistent with the HIP phenotype. In a separate experiment, five pairs (one from each phenotype) of female SD rats were co-housed. Prior to cohabitation, the phenotype was confirmed by NMR and after cohabitation of one week, urine was again collected and analyzed. Results indicated that after one week of cohabitation, the rats initially having the CA phenotype had converted to excreting urine with a metabolite profile consistent with the HIP phenotype, Figure 2. After three weeks of cohabitation, the rats were then housed individually and monitored for an additional two weeks. The urine metabolite profile remained consistent with the HIP phenotype with no reversion to the original phenotype in CA animals.

DISCUSSION

This report documents a consistent phenotypic difference within Sprague-Dawley (Crl:CD(SD)) rats supplied by the Charles River Raleigh facility that has remained stable for at least 12 months. Though a phenotype change as described here could be attributed to genetic drift, that is probably not the case here—at least in the usual sense. The changes observed in this report are most likely due to differences in the gut flora populating the GI tract. This is based primarily on two lines of evidence. Firstly, the urinary biomolecular differences observed in this study are consistent with previous reports of microflora-induced changes in urinary metabolites, particularly hippuric acid and chlorogenic acid metabolites (Gavaghan et al., 2001; Phipps et al., 1998). The second line of evidence is that when rats were housed in pairs, one from each phenotype, there was a relatively rapid switch of the phenotype of CA animals. This was most likely due to coprophagia inoculation of HIP-derived bacteria into the CA rats. The rapidity of the change ruled out an endogenous genetic component.
The GI tract is home to a vast host of microbiota with hundreds of species present in the normal mammalian GI tract (Guarner and Malagelada, 2003). Bacterial density varies with the topography of the GI tract with <1000 bacterial colony forming units (CFU) per ml present in the stomach to several hundred billion CFU/ml present in the colon (Hart et al., 2002). Comprehensive bacterial species identification of this complex ecology is difficult, if not impossible, particularly as speciation of the gut flora varies with species, age, and diet (Brennan-Craddock et al., 1992; Hebuterne, 2003; Rowland et al., 2000). However, an overview of the variety of gut flora populations in normal and disease states in humans has been published (Salminen et al., 1995). The role of gut flora in health is well recognized (Hart et al., 2002; Hooper et al., 1998, 2002; Hooper and Gordon, 2001; Simon and Gorbach, 1984; Stagg et al., 2004) with the fields of prebiotics and probiotics based on the concept of alteration of gut flora to treat disease or otherwise improve health (Gibson, 1998; Karimi and Pena, 2003). Therefore, it should not be terribly surprising that alterations in gut flora may cause fairly profound systemic effects. Nicholson and colleagues (Nicholson et al., 1998, 2002; Hooper and Gordon, 2001; Simon and Gorbach, 1984; Stagg et al., 2004) with the fields of prebiotics and probiotics based on the concept of alteration of gut flora to treat disease or otherwise improve health (Gibson, 1998; Karimi and Pena, 2003). Therefore, it should not be terribly surprising that alterations in gut flora may cause fairly profound systemic effects. Nicholson and colleagues (Nicholson et al., 1998, 2002; Hooper and Gordon, 2001; Simon and Gorbach, 1984; Stagg et al., 2004) with the fields of prebiotics and probiotics based on the concept of alteration of gut flora to treat disease or otherwise improve health (Gibson, 1998; Karimi and Pena, 2003). Therefore, it should not be terribly surprising that alterations in gut flora may cause fairly profound systemic effects. Nicholson and colleagues (Nicholson et al., 1998, 2002; Hooper and Gordon, 2001; Simon and Gorbach, 1984; Stagg et al., 2004) with the fields of prebiotics and probiotics based on the concept of alteration of gut flora to treat disease or otherwise improve health (Gibson, 1998; Karimi and Pena, 2003). Therefore, it should not be terribly surprising that alterations in gut flora may cause fairly profound systemic effects.

The authors implicated microfloral changes as an explanation for this lack of reversal. While we report similar results, they vary in some important respects. Firstly, the CA rats observed in these studies had measurable, though low, levels of hippuric acid. More importantly, the diet was not the source of the variation, in our studies since animals were obtained from the same facility with identical husbandry practices between rooms. Gavaghan et al. (2001) noted phenotypic variation in Sprague-Dawley rats consistent with that observed here in that they also noted phenotypes defined by high and low levels of hippuric acid instead of its presence and absence as reported by Phipps. The authors indicate that the phenotypes represent subpopulations but are unclear as to the source of their animals or the stability of these subpopulations.

As animals are born, raised, and distributed from a single room within the facility it is possible that after an initiating (and currently unexplained) event, gut floral changes occurred within the Room 9 population and have been passed from dam to weanling propagating the changes ever since. Another alternative explanation involves the sourcing of rats for Raleigh Room 9. The colony of rats maintained in Room 9 was originally started from foundation colony rats that had only eight species of bacteria, the Charles River Altered Schaedler Flora (Charles Clifford, Charles River Laboratory, personal communication). A detailed description of the Schaedler flora can be found in the article by Sarma-Rupavtarm and colleagues (Sarma-Rupavtarm et al., 2004). These “isolators” are maintained separately and used as a source for new colony initiation. Even after several years, these isolators have very limited number of gut bacteria species. Of the facilities we evaluated, Room 9 represented the most recent colony initiation in December 2002. The colony in P09 initiated in March 2000, while the colonies in K93 and K97 initiated in May 1997. As colonies are initiated and moved to barrier rooms for...
propagation, a repopulation of a “normal” gut flora may occur over time. In this scenario, the gut floral variation isn’t really a species shift, but is due to a lack of the “normal” speciation that occurs as rats are exposed to various bacteria, which evidently may be quite protracted. This scenario is supported by studies conducted by Nicholls et al. (2003) who monitored urine metabolic profile change in axenic Fisher-344 rats exposed to a normal laboratory environment to allow gut flora repopulation. They reported that initially low levels of urinary hippuric acid increased dramatically after 21 days of exposure to the normal laboratory environment. The primary argument against this scenario is the timing. If the colony in Room 9 had been initiated weeks or even months ago, this scenario would seem more likely. However, why would there be such a striking difference in a colony initiated three years ago versus one started five years ago? Nevertheless, this scenario deserves further investigation.

Of greatest concern with regard to these findings is what, if any, significance these phenotypic changes engender. These observations raise some interesting questions from a toxicologic perspective. The fact that these changes are not simply a reflection of diet-induced modulation of the microflora, but can persist for some time is highly significant. The gut flora are known to influence metabolism of numerous compounds which can profoundly affect toxification and detoxification pathways (Boxenbaum et al., 1979; Goodwin et al., 1994; Mikov, 1994; Rowland, 1981, 1988). The role of gut flora has been directly linked to the toxicity (or lack of toxicity) of numerous compounds including nitrobenzene, amygdalin, and digoxin to name a few (Mathan et al., 1989; Reddy et al., 1976; Rowland, 1988). Activation (or lack of detoxification) by gut flora can play a role in tumor induction by exogenous compounds (Rowland and Walker, 1983; Yoshida et al., 2003). If indeed the phenotypic changes are stable for even longer than the four weeks evaluated in this report, perhaps of even greater interest is what effect microflora changes may have on exposure to natural (i.e., dietary derived) metabolites (e.g., chlorogenic acid metabolites) and what effect these changes will have on background tumor incidence. If such differences in gut flora are persistent, they represent a potential lifetime exposure to relatively high concentrations of these varying metabolites. Could altered microflora be one explanation for what otherwise seems inexplicably fast drift in background tumor incidences? More generally, are these epigenetic variations hidden sources of variation in preclinical animal studies? These questions remain to be answered.

It is possible that these effects simply reflect a transient phenomenon that comes and goes within populations. Alternatively, rigorous efforts to maintain “clean” animal facilities may in some cases cause problems rather than avoid them. In either case the findings provide an opportunity to more fully understand the role of microflora in rat systems biology. As it is likely these changes have occurred from time to time in the past and are almost certainly characteristic of all laboratory animal suppliers, one could argue that they have had little effect on our ability to conduct and interpret toxicology studies. However, the truth of the matter is that we don’t know what we don’t know. It is plausible that anomalous findings that occasionally pop up in toxicology studies are not really anomalous but attributable to unrecognized microfloral changes. Likewise, the interstudy “variability” we have grown accustomed to in toxicology studies may not really be a biologic constant but rather may be at least partially attributable to definable causes, such as microfloral differences. What is undeniable is that the newer “omic” technologies are very sensitive to off target (systemic) changes. Therefore, it is imperative that studies using these technologies understand the nature of their animal models, including the role gut flora play. The complexity of life is a beautiful thing, though it certainly can be confusing sometimes. As new technologies delve ever deeper into systems biology, it can be anticipated that the more we learn, the more we will need to critically reassess our most basic assumptions.

In conclusion, we have identified a phenotypic difference in commercially supplied Sprague-Dawley (Crl:CD(SD)) rats that has been stable within a single room of the Charles River Raleigh facility for a period of at least 12 months. This phenotypic difference is most likely due to altered gut flora and is typified by diminished urinary hippuric acid and increased chlorogenic acid metabolites.

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REFERENCES


