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Identification of Urinary Biomarkers from X-Irradiated Mice Using NMR Spectroscopy

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In a major radiological event, rapid screening of radiation-exposed individuals for possible medical intervention is critical. Here we suggest a high-throughput, non-invasive approach to identify radiation biomarkers in urine and demonstrate a proof of principle in mice. Mice were whole-body irradiated (8 Gy X rays), and urine samples were collected from both irradiated and control mice for 7 days after exposure. ^{1}H nuclear magnetic resonance (NMR) spectra of all the urine samples were acquired on a spectrometer operating at a proton frequency of 600 MHz. The multivariate data were analyzed by principal component analysis (PCA). The resulting biomarkers revealed a broad range of metabolism changes, including creatine, succinate, methylamine, citrate, 2-oxoglutarate, taurine, N-methyl-nicotinamide, hippurate and choline. The temporal dependence of several biomarkers on radiation exposure was also explored. Combining several metabolomic biomarkers with different temporal dependence could provide an estimate of when the radiation exposure occurred. These results will be helpful in projecting metabolomic "fingerprints" in humans exposed to radiation.

INTRODUCTION

As the threat of terrorism increases globally, the possibility of a radiological attack in a public place is of great concern. The range of possible scenarios for such events is wide, with as few as zero and as many as tens of thousands of individuals potentially exposed to high doses of radiation. How best to address the health effects of such a radiation exposure (*1*) is a serious problem. In some situations it is clear that appropriate medical care can substantially shift the lethal dose at 60 days ($LD_{50/60}$) to higher doses (*2*). Thus, in the context of such an attack, rapid, early screening of exposed individuals will be critical to identify the individuals who need urgent medical intervention and to reassure (in most scenarios) the majority of individuals who received minimal or low radiation exposure.

A variety of methodologies have been employed to identify radiation exposure, such as the well-established cytogenetic analysis of peripheral blood lymphocytes (*3–5*), micronucleus assays (*6*), measurement of radiation-induced apoptosis (*7*), and serum proteomic analysis (*8*). These approaches are based on either blood cells or serum and, at least until recently (*9*), required that qualified health professionals draw peripheral blood through venipuncture. Such a procedure is potentially a major bottleneck in a mass casualty incident because a health professional can at most draw blood from 15 to 25 individuals per hour. Again until recently (*9*), these technologies required both comprehensive analysis by skilled workers as well as considerable time (*8*). Thus there is a pressing need for new high-throughput non-invasive biodosimetry methodologies for large population screenings (*9*).

Metabolomics, the study of small molecules (metabolites) involved in biochemical processes in a living system, is one such method, because it provides a means of screening large populations in a relatively short time (*10, 11*). Furthermore, if it can be carried out using urine rather than blood, samples can be collected more easily and rapidly and potentially analyzed rapidly and inexpensively (*12*). A number of recent studies have shown that urine is a good candidate for metabolomics studies (*13–17*). The present work was designed to identify radiation biomarkers in the urine of radiation-exposed mice.

Two technologies dominate metabolomics studies: mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. As reviewed elsewhere (*18, 19*), both technologies have advantages and disadvantages. While the high sensitivity of MS is attractive, the advantages of NMR for metabolomics studies are its nonselectivity, lack of sampling bias, and reproducibility (*18*). Moreover, NMR technology requires minimal

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sample preparation and a relatively short time to obtain a spectrum. Therefore, this high-throughput technique enables us to analyze a large number of samples within a relatively short time.

Recent work using MS has identified some biomarkers associated with γ radiation in rodents by employing MS-based derivatives (*14, 15, 17*). Complementary to this work, we present here the results of a study of urinary metabolomic biomarkers from X-irradiated mice using NMR spectroscopy. This approach, based on detection of a profile of radiation-induced small molecules by NMR, has the potential to have extremely high throughput. By using this combination with MS, we should be able to measure metabolomic ''fingerprints'' in humans exposed to radiation.

MATERIALS AND METHODS

Animals

All animal handling and experimental protocols were reviewed and approved by the Institute of Comparative Medicine at Columbia University Medical Center (Protocol number AC-AAAA7174). Male C57BL/6 mice, which were chosen for their intermediate radiation sensitivity, aged 6 weeks were purchased from Taconic Farms, Inc. (Germantown, NY) and housed in the local animal housing facility for 2 weeks on a standard 12/12-h light-dark cycle to allow them to adjust to the new environment. The mice were given regular laboratory chow and water *ad libitum* (*14*).

Acclimatization and X Irradiation

Before irradiation, it was important to acclimate (*14*) the mice to the radiation and urine collection protocol, which consisted of being placed in 50-ml polypropylene Falcon tubes (Becton Dickinson, Franklin Lakes, NJ) used to immobilize the mice during irradiation as well the housing in the metabolic cages used to collect urine. These steps were necessary to eliminate the effect of stress on the urinary metabolome due to environmental changes (*14*). Three days prior to irradiation, the mice were placed in individual Nalgene metabolic cages (Tecniplast USA, Inc., Exton, PA) for 6 h per day as well as in Falcon tubes fitted with an air-transmitting plug for 10-min periods twice a day. Eight of the 12 mice used in this study were irradiated individually in Falcon tubes with a whole-body dose of 8 Gy 250 kVp X rays at a dose rate of 3.7 Gy/min. The $LD_{50/30}$ for C57BL mice is in the range of 6–8 Gy (*14, 20, 21*); we used 8 Gy in this study to maximize any potential metabolomic response.

Urine Collection

After irradiation, the urine samples were collected from both irradiated and control mice held in individual metabolic cages without food but with *ad libitum* water for a 6-h period each day for 7 days. The collection procedure was conducted at the same time each day (10:00 a.m.–4:00 p.m.) throughout the study to eliminate diurnal variations in the urine. For the remainder of the time they were kept in regular cages with their littermates and with food and water *ad libitum*. Food was not provided in the metabolic cages to avoid physical contamination of the urine samples. To evaluate the effect of food deprivation on the mouse urinary metabolome, we collected urine for 5 days in a preliminary control experiment. The results showed that food deprivation for 6 h a day had no apparent effect. One of the mice died 3 days after exposure to radiation; only three urine samples were collected for this mouse. A total of 80 urine samples were collected from the 12 mice and ranged in volume from 0.1 to 1.6 ml. The urine samples were stored at $-80^{\circ}C$ until use.

NMR Samples and Spectroscopy

For the urine samples with volumes equal or greater than 0.3 ml, aliquots of 0.3 ml urine were mixed with 0.3 ml phosphate buffer (0.2 *M*, pH 7.4). For those with volumes of less than 0.3 ml (14 samples), phosphate buffer was added to the samples until the total volume was 0.6 ml. Any precipitate was removed by centrifugation. For each sample, 540 µl of supernatant was transferred to a 5-mm NMR tube and mixed with 60 µl of sodium 3-trimethylsilyl-(2,3,3,3-D4)-1-propionate (TSP)/D_2O/sodium azide solution (5 m*M* TSP and 1% w/v sodium azide in 100% D_2O). NMR spectra were acquired at 300 K on a Bruker Advance DMX spectrometer operating at a proton frequency of 600 MHz using a Cryo probe. All urine samples were prepared and acquired in a random order to avoid introducing human and instrument bias into the data classification. The excitation sculpting pulse was used for water suppression in the NMR experiments (*22*).

Data Processing and Statistical Analysis

Informatics data analysis was performed using the comprehensive software tool *HiRes* that was developed in our laboratory (*23*). All the spectra were loaded to the *HiRes* software (*23*), and five spectra were removed from the analysis due to broad resonances caused by either samples being too diluted or poor shimming. The spectra were first corrected for phase and baseline distortions and then globally aligned to TSP (0 ppm reference). The intensity of creatinine peak at 4.06 ppm was used to normalize the intensities of individual peaks for each spectrum. Therefore, the concentrations of individual metabolites are relative to creatinine on a per sample basis. Creatinine is commonly used as a normalization reference in metabolomics studies (*14, 24*) because it is a good measure of renal function in individual animals and thus, to first order, eliminates individual difference in water reabsorption (*25*). For peaks that are very sensitive to the small variation in pH and salinity of the solution (for example, citrate), local alignments were applied (*26*). The multivariate data were analyzed by using principal component analysis (PCA), which can transform a large number of correlated variables into a smaller number of uncorrelated variables (principal components). PCA results have two complementary parts: principal components (PCs) and corresponding score plots. Generally, the first PC represents the maximum variation in the data while each successive PC accounts for as much of the remaining variability as possible. The PC score plot indicates the relative magnitude of individual study subjects. Four components were calculated in our work and represented approximately 90% of the total variance. Once PCA identified possible biomarkers, the mean PC scores representing the relative magnitude of individual biomarkers were tested for difference between control and irradiated groups by a two-tailed *t* test assuming unequal variances. Only those with 95% confidence ($P < 0.05$) were chosen as biomarkers of radiation. Since we were testing specific compounds identified through the PCA analysis, no multiple comparison correction was made.

RESULTS

A representative ^1H NMR spectrum of a mouse urine sample from a 600 MHz spectrometer and the assignments of major resonances are shown in Fig. 1. The spectral region (δ 4.5–6.4) containing the residual water and large urea peaks was excluded from analysis. Spectral assignments were made from literature values (*13, 27, 28*) and the online Human Metabolome Database (*29*).

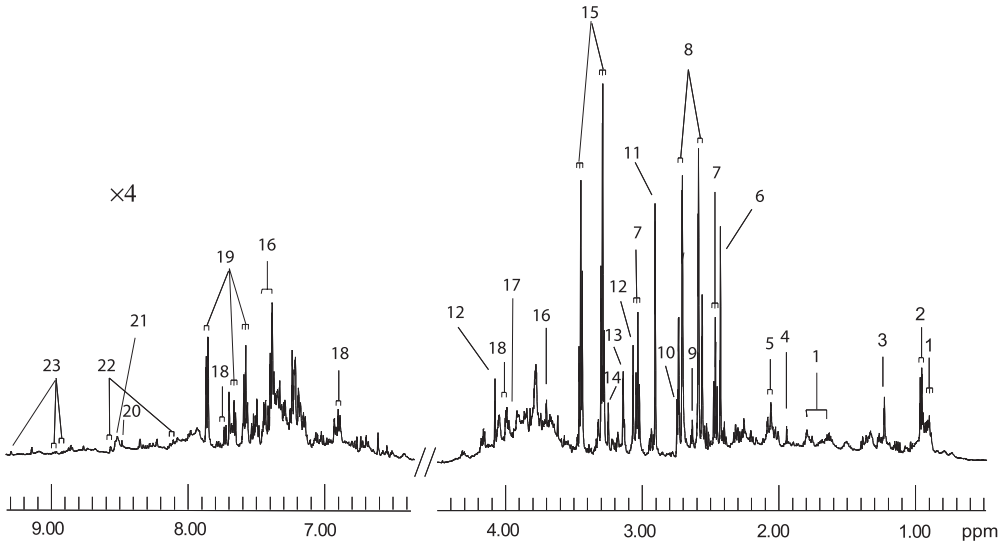

FIG. 1. A typical 600 MHz ^{1}H NMR spectrum of mouse urine. The spectral region (δ 4.5–6.4) containing imperfect water suppression and large urea peak was removed for display and analysis. The spectra in the aromatic region (δ 6.4–9.3) were magnified four times compared with the region δ 0.5–4.5. Assignments: 1, 2-hydroxybutyrate; 2, 2-oxoisocaproate; 3, unknown singlet; 4, acetate; 5, N-acetyl group; 6, succinate; 7, 2-oxoglutarate; 8, citrate; 9, methylamine; 10, dimethylamine; 11, trimethylamine; 12, creatine; 13, malonate; 14, choline; 15, taurine and trimethylamine-N-oxide; 16, phenylacetylglycine; 17, creatinine; 18, 4-aminohippurate; 19, hippurate; 20, formate; 21, N-methyl-4-pyridone-5-carboxamide (4PY); 22, N-methylnicotinate (NMN acid); 23, N-methylnicotinamide (NMN amide).

PCA analysis was applied to individual regions consisting of resonances with comparable intensities so that higher-intensity peaks would not overwhelm those with lower intensities. Figure 1 shows a typical spectrum with most resonances in the downfield region (δ 6.4–9.3) and with much lower intensities than those in the upfield region (δ 0.5–4.5).

In this study, the mice produced the highest volume of urine and were very active on the first day of acclimatization and were under considerable stress as they adjusted to the new environment on their first day in the metabolic cages. To determine whether 3 days of acclimatration was sufficient to eliminate the effect of stress on the metabolic profile, we analyzed the NMR spectra of urine from the four control mice (ten urine samples for each mouse including three from the acclimatization period) by PCA (not shown). The results indicated that a few metabolites showed the largest variation on the first day of the acclimatization and that the level returned to baseline by the fourth day in the metabolic cage. Therefore, 3 days of acclimatization is both necessary and long enough for mice to adjust to the stress caused by handling and caging.

In the upfield spectral region (δ 0.5–4.5), taurine, citrate and trimethylamine are present at relatively high concentrations in urine. In addition, as shown in the NMR spectrum (Fig. 1), one of the two NMR triplets representing taurine (δ 3.24, δ 3.27) overlaps with that of trimethylamine-N-oxide (TMAO) (δ 3.27). Therefore, in this region, the PCA was first performed on the spectra with the resonances of taurine, TMAO, trimethylamine and citrate excluded. The first two PCs and corresponding score plot for this upfield region are shown in Fig. 2A and B, respectively. The PCA analysis provided good separation between irradiated and control mice (Fig. 2B). The separation is mostly from the second PC (along Y axis in Fig. 2B); specifically, the PC2 scores are lower for the irradiated mice than for the control mice. Therefore, the second PC appears to choose promising radiation biomarkers. The possible biomarkers chosen in this region are acetate, succinate, 2-oxoglutarate, methylamine, creatine, choline and a singlet at 1.21 ppm (Fig. 2A). Further evaluation of these metabolites individually by a t test indicated that acetate levels were not significantly different between the control and irradiated groups ($P > 0.05$) and therefore disqualified acetate as a biomarker. The rest were confirmed by t tests as real biomarkers that responded to the radiation ($P < 0.05$). Among these biomarkers, the urinary levels of creatine and choline were increased while the rest (succinate, 2-oxoglutarate and methylamine) were decreased in the irradiated mice compared with the control group. To our knowledge, assignment of the singlet at 1.21 ppm has not been reported in the literature. Identifying this metabolite is clearly important, and related 2D NMR and spike experiments are ongoing.

The downfield region of the NMR spectra (δ 6.4–9.3) consists of resonances from metabolites with aromatic rings. Within this aromatic region, the part δ 8.15–9.30 mainly consists of resonances from N-methyl-nicotinate (NMN acid), N-methyl-nicotinamide (NMN amide), N-methyl-4-pyridone-5-carboxamide (4PY) and formate,

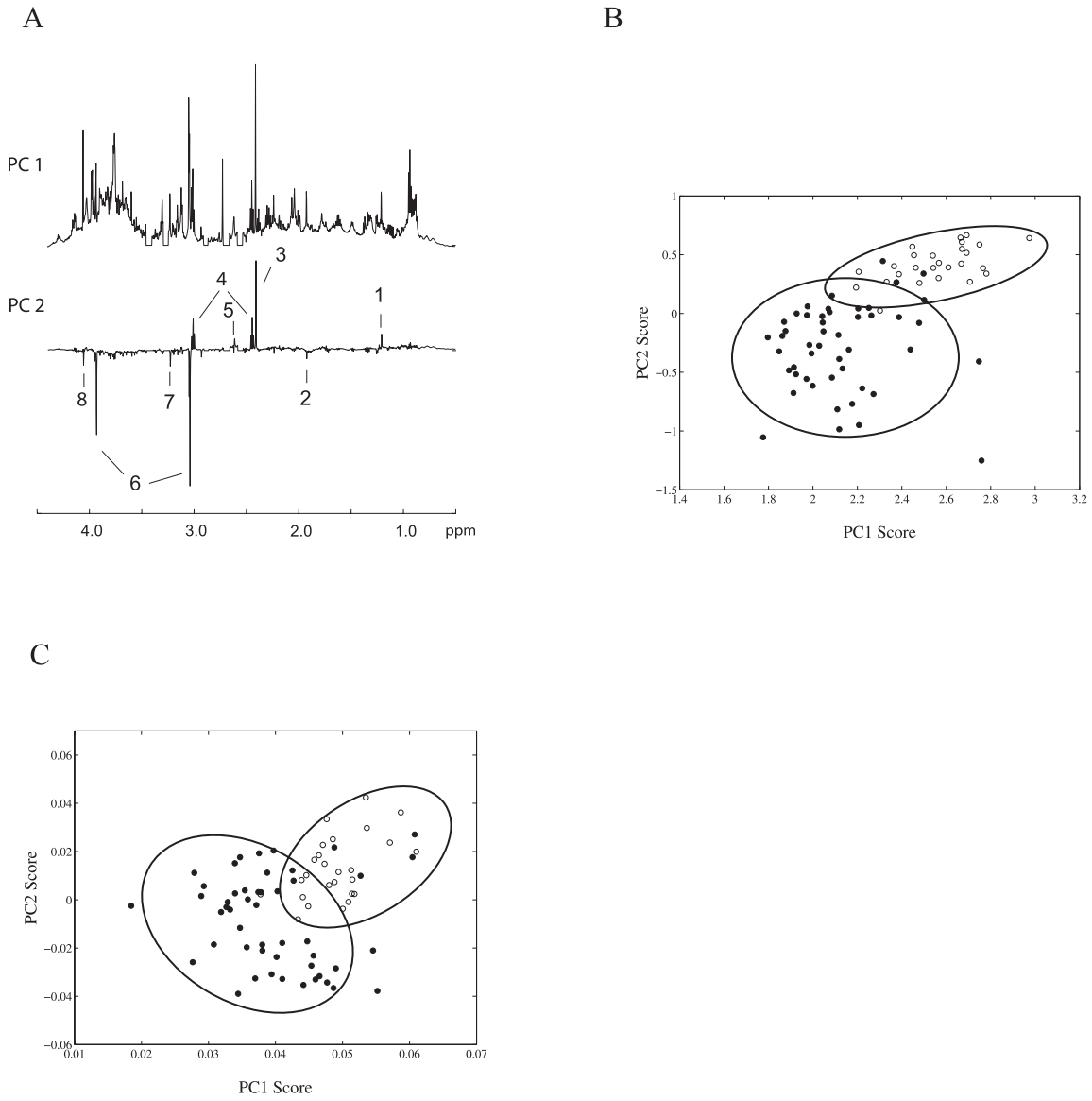

FIG. 2. The first two PCs (panel A) and the corresponding score plot (panel B) of PCA from ^{1}H NMR spectra of urine from irradiated (filled circles) and control mice (open circles) for the upfield region (δ 0.5–4.5) after resonances from taurine, TMAO, trimethylamine and citrate were removed. Metabolites identified from PC2 only: 1, unknown singlet; 2, acetate; 3, succinate; 4, 2-oxoglutarate; 5, methylamine; 6, creatine; 7, choline; 8, creatinine. Panel C: The PC1 vs. PC2 plot of PCA for the aromatic region (δ 8.15–9.30).

which are present at extremely low concentrations in urine. To identify potential biomarkers in this region, PCA was performed on this part (δ 8.15–9.30) separately. The resulting plot of PC1 vs. PC2 is shown in Fig. 2C. Although there are several outliers, separation between irradiated and control mice is clearly significant. Because of spectral noise and distorted baseline issues, the only biomarker we could unquestionably identify in this region was NMN amide. The rest (δ 6.4–8.15) of the aromatic region was dominated by a few metabolites, including hippurate and tryptophan; analysis of this region identified no obvious biomarkers that could be used to distinguish the irradiated mice from the control group, although the hippurate level had a complex time dependence after irradiation that we will discuss below.

For taurine, TMAO, trimethylamine and citrate, PCA was applied individually to the resonances from each molecule. The urinary level of trimethylamine varied among the mice but showed no significant separation between irradiated and control mice. Analysis of citrate revealed an interesting separation pattern between irradiated and control mice. Specifically, the levels of citrate in urine from irradiated mice decreased after irradiation and reached a minimum on the fourth day postirradiation. From the fifth day, the levels started to recover, and by the sixth and seventh days postirradiation, the levels in urine from irradiated mice were comparable to and in some cases higher than in urine from control mice. In this case, the typical two-dimensional PC1 vs. PC2 score plots would obscure those patterns. To elucidate this temporal

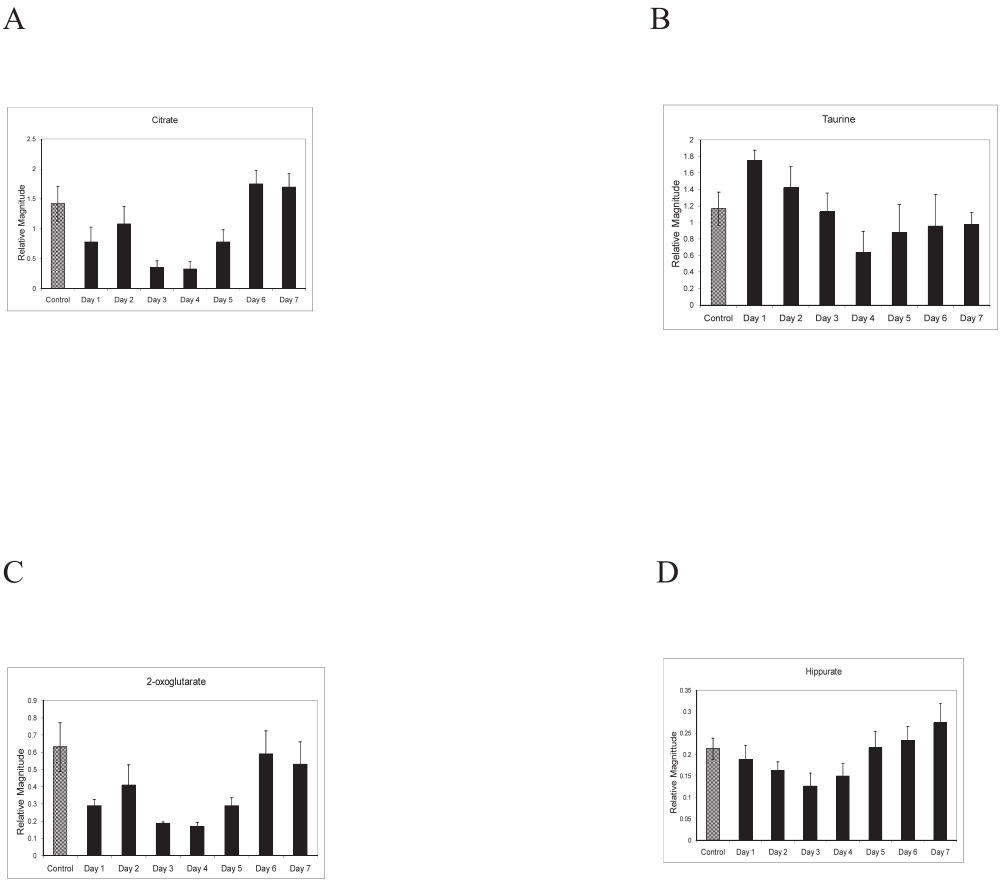

FIG. 3. Relative magnitude on average representing citrate (panel A), taurine (panel B), 2-oxoglutarate (panel C) and hippurate (panel D) in urine samples from control mice (gray bar) and irradiated mice (black bars) for different times postirradiation.

dependence, the mean first PC score, which represents the magnitude of the resonances, was plotted for different postirradiation times for the irradiated and control mice (Fig. 3A). For taurine, we used only one triplet (δ 3.24) for the analysis, because this completely represents the concentration of taurine in urine. The analysis revealed that taurine was moderately elevated on the first day after irradiation but subsequently returned to baseline (Fig. 3B).

To evaluate the temporal dependence of the other biomarkers, detailed PCA was applied to the metabolites individually. The results suggested that 2-oxoglutarate and hippurate also exhibited significant temporal dependence. The magnitudes of 2-oxoglutarate and hippurate for different postirradiation periods compared to control are shown in Fig. 3C and D, respectively. 2-Oxoglutarate demonstrated temporal dependence that was very similar to that of citrate; the magnitude decreased significantly after irradiation and then recovered by 5 days postirradiation (Fig. 3C). On the other hand, the level of hippurate did not show a significant decrease until the third day postirradiation (Fig. 3D). Overall, the urinary levels of all three metabolites (citrate, 2-oxoglutarate and hippurate) reached a minimum on the third and fourth days postirradiation and returned to the baseline on the sixth and seventh days.

A summary of all the urinary biomarkers that had uniform responses to radiation is shown in Table 1. A summary of the urinary biomarkers that showed a temporal dependence is displayed in Table 2 along with their individual responses 7 days postirradiation.

DISCUSSION

The current work demonstrates the potential of urine NMR spectroscopy and analysis to provide a noninvasive methodology capable of rapidly identifying radiation-exposed individuals in a population. The urinary biomarkers identified so far are either end products or intermediates from various metabolism pathways: succinate, citrate and 2-oxoglutarate from energy metabolism via the tricarboxylic acid cycle (TCA cycle), methylamine from amine metabolism, choline from lipid metabolism, creatine and taurine from amino acid metabolism, and NMN amide from nucleotide metabolism. This wide range of altered metabolites indicates that whole-body exposure to 8 Gy X radiation disturbs a number of living functions.

TABLE 1
Identified Metabolites that Have Uniform Change in Urine from Irradiated Mice Compared with Control Mice

Metabolite	Chemical shift in ppm and multiplicity	Change direction [a] ($P < 0.005$)
Creatine	3.93 (s), 3.04 (s)	↑
Succinate	2.41 (s)	↓
Unknown	1.21 (s)	↓
Methylamine	2.61 (s)	↓
N-Methyl-nicotinamide	8.97 (d), 9.28 (s), 8.90 (d)	↑
Choline	3.22 (s), 3.51 (m), 4.07 (m)	↑

[a] ↑ significantly elevated, ↓ significantly reduced.

Comparison of our results with the biomarkers identified using MS (*14, 15, 17*) shows a few commonalities of the technologies. Employing gas chromatography-mass spectrometry (GCMS), Lanz *et al.* (*17*) indentified 11 urinary biomarkers in rats irradiated at a lower dose (3 Gy). Of these biomarkers, downregulated citrate and 2-oxoglutarate were detected, as was the case in the present study. Based on ultra-performance liquid chromatography-time-of-flight mass spectrometry (UPLC-TOFMS), Tyburski *et al.* (*14*) identified distinct urine metabolomic phenotypes from mice irradiated with 3 and 8 Gy. However, only one common biomarker was identified in their study and ours: elevated taurine levels in mice irradiated with 8 Gy. The urine collection protocols in the MS studies were significantly different from ours (*14, 15, 17*). In those studies, the urine samples were collected after the mice had been in the metabolic cages for only 24 h. Since the changes in some metabolites may peak at certain times, it is possible that some changes would not be apparent in the 24-h urine. Further, in contrast to the present study, *ad libitum* food was provided to the mice in the MS studies. Both differences may complicate a comparison of urinary metabolomic "fingerprints" in these studies. The UPLC-TOFMS study examined only the first 24 h after radiation exposure (*14*), while we followed the mice for 7 days. Another UPLC-TOFMS study by the same research group (*15*) extended the study to 4 days, although at a lower radiation dose (1–3 Gy), and found results similar to those for the shorter time. Although it is possible that some metabolites may have been missed by UPLC-TOFMS because of the different time scales in the studies, most of the biomarkers reported here showed significant changes even on the first day after exposure, suggesting that the different time scales are not the main reason for this difference. As pointed out by Lanz *et al.* (*17*), many of the highly polar metabolites present in urine are not detected by TOFMS because they elute in the first minute of the UPLC chromatogram. Therefore, we were able to identify several urinary biomarkers that escaped detection by UPLC-TOFMS due to their polarity. However, many biomarkers identified by UPLC-TOFMS have very low abundance in urine and are effectively invisible to NMR because of its lower sensitivity. These results suggest that the complementary use of MS and NMR may provide the most comprehensive metabolomic results.

Tyburski *et al.* (*14*) observed that the change in urinary taurine levels was not statistically significant after 3 Gy irradiation but that taurine increased 20% after 8 Gy irradiation. Our results (Fig. 3B and Table 2) indicate that the urinary taurine levels increased significantly on the first day after 8 Gy irradiation but returned to the baseline level thereafter. These findings suggest that taurine could serve as an early biomarker of high-dose radiation exposure. Taurine and the corresponding taurine transporter play important roles in brain, retina and kidney development (*30*). Higher taurine has been observed in mice with radiation-induced brain injuries (*31*). It has also been suggested that increased excretion of taurine may arise from altered renal reabsorption of taurine due to reduced glomerular filtration rate (GFR) or possibly as a general stress response to an external stimulus (*28, 30*). We normalized the intensities of the individual spectra to creatinine, which should eliminate any individual differences in GFR (*25*). It has also been reported that 8 Gy radiation had no apparent effect on the total production and excretion of creatinine (*14*). *In vitro* experiments have shown that ischemia induces taurine release in the developing mouse hippocampus (*32*). The increase in extracellular levels of taurine was a response to preserve cell integrity under damaging conditions (*32*), and it may serve as an important protective mechanism against excitotoxicity (*30*). Thus we conclude that elevated taurine in the urine of irradiated mice at an early stage is most likely a stress response to the high dose of radiation and

TABLE 2
Changes in Metabolites in Irradiated Mice that Have Temporal Dependence Compared with the Control Mice

Metabolite	Chemical shift in ppm and multiplicity	Change in direction in 7 days [a,b]						
		1	2	3	4	5	6	7
Citrate	2.70 (d), 2.56 (d)	↓*	↓**	↓*	↓*	↓*	→	→
2-Oxoglutarate	2.45 (t), 3.01 (t)	↓*	↓**	↓*	↓*	↓*	→	→
Taurine	3.42 (t), 3.27 (t)	↑*	→	→	↓*	→	→	→
Hippurate	7.84 (d), 7.55 (t), 7.64 (t), 3.98 (d)	→	↓*	↓*	↓*	→	→	↓**

[a] ↑ significantly elevated, ↓ significantly reduced, → no statistically significant change ($P > 0.05$).
[b] *$P < 0.005$, **$P < 0.05$.

may reflect an important protective mechanism against acute radiation damage.

Most of the biomarkers identified by NMR exhibited reduced levels after radiation exposure, with the most significant reductions seen in those metabolites involved in energy metabolism, i.e., succinate, citrate and 2-oxoglutarate. Dramatically reduced levels of citrate and 2-oxoglutarate have been observed in rats exposed to 3 Gy γ radiation (*17*), which is in agreement with our observations. In the earlier study, it was concluded that the reduced levels of citrate and 2-oxoglutarate were caused by a decline in renal tubular energy production rather than low caloric intake (*17*). Yushmanov (*33*) found a significantly reduced level of citrate in urine samples from the individuals exposed after the Chernobyl reactor accident. Succinate, citrate and 2-oxoglutarate are products from the same metabolism pathway, the TCA cycle. Taken together, these results imply that this high dose of radiation has a significant effect on the energy metabolism of mice and that the urinary levels of succinate, citrate and 2-oxoglutarate could serve as specific biomarkers of past radiation exposure in mammals.

The most dramatic elevation of a biomarker in urine postexposure was that of creatine. Before irradiation, the resonances of creatine were small, almost within the baseline resonance. On the first day postexposure, the intensities of the peaks increased significantly. On the third and fourth days, the intensities of the peaks reached a maximum, and the average intensity of the peaks was fourfold higher compared to the control group. The metabolism of creatine has been reviewed extensively (*34*): in mammals, approximately half of the required creatine comes from diet (mainly from meat) and half is synthesized from three amino acids: arginine, glycine and methionine. The biosynthesis of creatine occurs mainly in the liver and the kidneys, and most of it is stored in muscle and functions as part of the cellular energy shuttle. The relationships between disturbances in creatine metabolism and human disease are not fully understood and are beyond the scope of our study. The greatly elevated urinary excretion of creatine in the irradiated mice is likely to be due either to excessive creatine production or to the inability of the muscles to take up all the available creatine. In line with the fact that the reduced levels of the metabolites involved in the TCA cycle (i.e. succinate, citrate, 2-oxoglutarate) were observed in the irradiated mice, the latter is more likely, and a radiation-induced slowdown in energy metabolism may be at least partly responsible for excessive urinary excretion of creatine.

The concentration of hippurate decreased in the urine samples from the irradiated mice (from the second day to the fourth day) compared with those from the control mice (Fig. 3D). Hippurate is a microbial metabolite, and its concentration is related to gut microbial activity. Studies have suggested that the variation of the urinary hippurate concentration in rats may be linked to changes in the distribution of intestinal flora (*16, 35*). Similarly, the change in urinary hippurate levels in the irradiated mice suggests gut microbial changes caused by high-dose radiation.

As pointed out in the Results section (and see Table 2), several biomarkers demonstrate a complex temporal dependence on radiation exposure, particularly citrate and 2-oxoglutarate (Fig. 3A and C). Time-dependent excretion of some metabolites (deaminated purines and pyrimidines) has also been observed in mice exposed to lower radiation doses (*15*). Succinate, another metabolite originating from the same TCA cycle as citrate and 2-oxoglutarate, displayed a similar change pattern on the first 5 days after exposure but different patterns on the sixth and seventh days; i.e., it stayed at lower levels all the 7 days. The mechanism(s) of these different time dependences awaits further clarification. However, we postulate that combining several biomarkers with different temporal dependences would make it possible to estimate when the radiation exposure occurred. A 3D plot of PC1 scores from PCA of succinate, citrate and NMN amide is shown in Fig. 4. The combination of these three biomarkers clearly reveals three distinguishable clusters: the nonirradiated control group, the third to fifth days after irradiation, and the sixth and seventh days after irradiation. Those from relatively short times postexposure (first and second days) are scattered within the first two groups, which may reflect the relative sensitivity of individual mice to radiation.

One of the most often cited criticisms of metabolomics studies is the problem of the "usual suspects"; namely, there are always some metabolites that respond to environmental stimulus or internal toxicant administration, regardless of nature of the stimulus, its mechanism of action, or its target (*18*). Most of the urinary biomarkers identified so far in our work are on such a list, including taurine, creatine, succinate, citrate, 2-oxoglutarate and hippurate. Therefore, it is possible that the changes in these metabolites are not specific to acute radiation exposure. For example, a recent study of urinary metabolic perturbations associated with acute liver toxicity indicated that the urinary levels of taurine and creatine increased while those of succinate, 2-oxoglutarate, citrate, hippurate decreased in the first 72 h after rats were given root of *Dioscorea bulifera L.* (*36*). These metabolic responses are very similar to those induced by acute radiation exposure. Nonetheless, it is also widely accepted that the magnitude, direction and temporal response of the changes among these "usual suspects" are different in individual systems; thus they are still useful in providing mechanistic or biomarker data as long as the changes are evaluated in the context of the systemic effect (*18*).

In addition to the "usual suspects" discussed above, some other metabolites have been identified as radiation biomarkers: methylamine, choline and NMN amide. The urinary level of methylamine decreased while those of choline and NMN amide increased after irradiation. Methylamine is a metabolite from amine catabolism, and

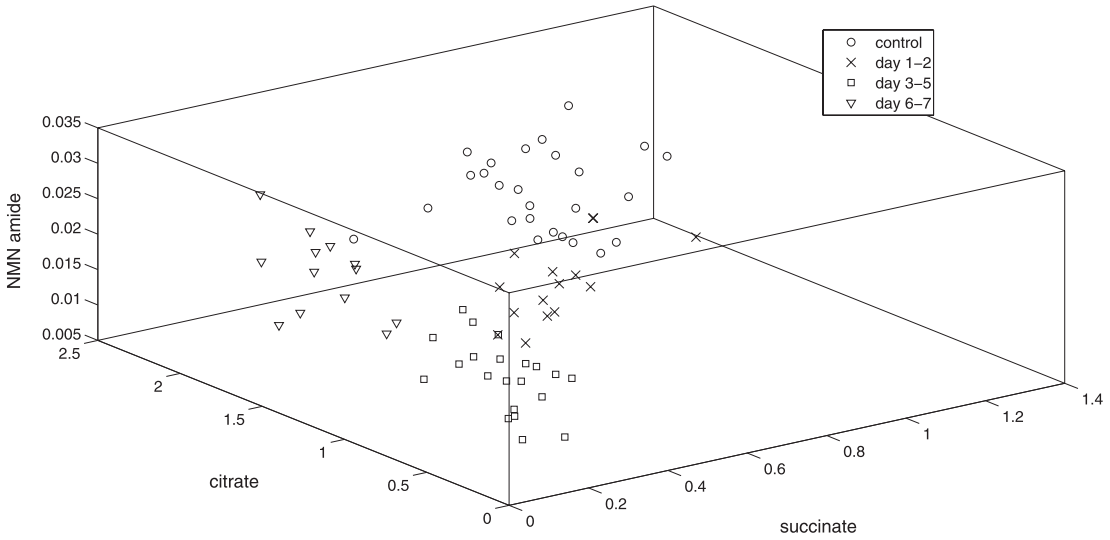

FIG. 4. Three-dimensional display of PC1 scores for citrate, succinate and NMN amide in the urine samples from the irradiated and control mice. The different symbols represent urine samples at different times postexposure.

its changed level may be associated with some pathological conditions (*29*); methylamine also targets the voltage-operated neuronal potassium channels and is probably involved in the release of neurotransmitters (*29*). Choline is essential in the central nervous system as a precursor for acetylcholine and membrane phosphatidylcholine. It is a major source of the methyl group for various metabolic processes and is involved in lipid metabolism. In a recent large population-based study, a significant inverse association was found between plasma choline concentration and anxiety levels (*37*). The NMR resonances representing NMN amide are very small due to its extremely low abundance in urine. Nevertheless, the PCA analysis indicated that the level of NMN amide increased considerably after irradiation. NMN amide in the urine involves nucleotide metabolism and may be an indicator of liver function. A rise in urinary NMN amide was observed in patients with liver cirrhosis and may have been caused by a broad impairment of liver oxidative metabolism (*38*). The elevated levels of NMN amide in urine from the irradiated mice suggests disturbed liver oxidative metabolism caused by radiation exposure.

While a broad range of metabolites have been identified as being associated with radiation exposure in the present study, NMR spectroscopy has its own limitations in a real large-scale nuclear event. First, operation of an NMR instrument requires well-trained personnel. Second, the size and highly demanding maintenance of high-field magnets make it impossible to use in the field. In this regard, MS is a better choice because a portable handheld MS is available commercially. However, simple sample preparation, easy data interpretation, and high reproducibility make NMR spectroscopy an excellent candidate for high-throughput screening in more remote locations.

In summary, we used NMR to identify biomarkers in mouse urine after exposure to 8 Gy whole-body X radiation. Changes in these biomarkers were detected on the first day after irradiation, and they typically reached their maximum on the third and fourth days after the irradiation. The responses of mice to a high radiation dose were characterized by broad ranges of metabolism changes, including slowed energy metabolism, damaged liver and kidneys, and altered gut microbes. Among the many biomarkers identified in the present work, urinary levels of succinate, citrate and 2-oxoglutarate appear to be excellent candidates for urine-based radiation biomarkers. The temporal dependence of several biomarkers on radiation exposure was also explored. Combining several biomarkers with different temporal dependence could provide useful information on when the radiation exposure occurred. This NMR-based work, along with the studies based on MS (*14, 15, 17*), provides a near-complete profile of radiation metabolomics in the rodent model. These data will be helpful to guide studies of metabolomic "fingerprints" in humans exposed to radiation.

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