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NMR-based metabolomics of urine for the atherosclerotic mouse model using apolipoprotein-E deficient mice

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Abstract

NMR-based metabolomics of mouse urine was used in conjunction with the traditional staining and imaging of aortas for the characterization of disease advancement, that is, plaque formation in untreated and drug-treated apolipoprotein-E (apoE) knockout mice. The metabolomics approach with multivariate analysis was able to differentiate the captopril-treated from the untreated mice in general agreement with the staining results. Principal component analysis showed a pattern shift in both the drug-treated and untreated samples as a function of time that could possibly be explained as the effect of aging. Allantoin, a marker attributed to captopril treatment was elevated in the drug-treated mice. From partial least squares–discriminant analysis, xanthine and ascorbate were elevated in the untreated mice and were possible markers of plaque formation in the apoE knockout mice. Several additional peaks in the spectra characterizing the study endpoint were found but their respective metabolite identities were unknown.

Keywords

NMR; ¹H; metabolomics; apoE; mouse; atherosclerosis

Introduction

Cardiovascular disease is the major cause of death in most developed countries and in many developing nations worldwide.^[1] The prevalence of this disease and its underlying atherosclerosis are correlated to LDL cholesterol commonly associated with the high-fat diets found in Western cultures.^[2] Because it is not readily feasible to obtain human atherosclerotic tissue to study the biochemical basis of atherosclerosis under different laboratory conditions, animal models have been developed in an attempt to mimic aspects of the human disease.^[3] Animal models have been further refined with the introduction of genetically engineered animals.^[4–7] We present results using the well-characterized apolipoprotein-E (apoE) receptor-deficient mice as a model of plaque formation^[8,9] and its inhibition. The control group was untreated and developed plaques. The drug-treated group was given captopril, an inhibitor of angiotensin I-converting enzyme, that is known to result in reduced plaque formation in this mouse model.^[10] The course of the study was 15 weeks;

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Supporting information

Supporting information may be found in the online version of this article.

at the end of which, the animals were sacrificed and *en face* analyses of the aortas were performed. Our goal with the study was to evaluate NMR-based metabolomics of mouse urine since it is relatively easy to collect and the same animals could then be monitored during the whole 15-week period with the hope of observing metabolic markers of disease progression or drug treatment.

Experimental

All the procedures involving animals were consistent with the guidelines (*Guide for the Care and Use of Laboratory Animals*) published by the National Research Council August, 2001, and approved by the animal ethics committee (Institutional Animal Care and Use Committee) at Johnson & Johnson Pharmaceutical Research & Development, LLC. Mice were housed under controlled conditions (20–22 °C; 50–65% relative humidity and 12 h light cycle) with access to food and water as described in the following text. There were 16 apoE^{-/-} mice with a C57BL/6 background used for the study, which were split into two groups – captopril-treated and untreated. The drug-treated group was given captopril (Sigma, St. Louis, MO) via the chow at a targeted dose of 5 mg/kg/day. Animals were ear tagged to be able to assign urine samples throughout the course of the study. Both groups of animals were fed the Harlan Teklad (Madison, WI, USA) TD-98338 chow, a ‘Western diet’ high in fat but with decreased cholesterol. The high-fat diet was used to hasten the plaque formation as the apoE mice will form plaque over the course of about a year when fed a standard chow diet. The mice were separately group-housed by treatment class until the time for urine collection and then they were placed in individual metabolism cages with free access to water but without food. Urine from all the mice was collected at the ends of weeks 2, 4, 6, 8, 10, 12 and 14 during an overnight 14–17-h collection period into tubes containing 100 µl of 10% sodium azide. The volume collected was usually about 1 ml/mouse. If feces contaminated the urine – a rare occurrence – this was noted. Samples were spun at 16 000 g in a microfuge for 3 min at 4 °C to remove precipitates and supernatants were transferred to labeled microfuge tubes and stored at –70 °C. The animals were always returned to their group-housing arrangement after the urine collection. Nonfasting glucose was measured just prior to sacrifice from a tail vein sample using the OneTouch Ultra glucose meter (LifeScan, Inc, Milpitas, CA).

For the quantitation of atherosclerosis, mice were anesthetized and bled from the inferior vena cava. Perfusion and *en face* preparations of Sudan IV-stained aortas were done as described.^[11–14] Images of the stained aortae were captured with a SPOT-RT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI), and morphometric image analyses were performed using Image-Pro image analysis software (Media Cybernetics, Inc., Bethesda, MD). The amount of atherosclerosis for each mouse was quantified by expressing the area of Sudan IV-stained lesion as a percentage of the entire area of the vessel (from the aortic arch to the iliac bifurcation) for each *en face* preparation examined. The values for this quantitation are found in the ‘% plaque’ column of Table 1. Atherosclerosis was quantified in a blinded manner such that the investigator performing the analyses was unaware of the treatment group from which each sample originated.

To prepare the urine for NMR analysis, 225 μ l of 0.2 M, pH 7.4 phosphate buffer was added to 225 μ l aliquots of the mouse urine along with 50 μ l of a 1 mg/ml solution of TSP (3-trimethylsilylpropionic-(2,2,3,3- d_4)-acid) in D_2O . The buffer was used to minimize chemical shift variation due to differences in the urine pH and the TSP served as a chemical shift reference. Samples were placed in 5 mm NMR tubes and NMR data were acquired on a Bruker DMX-600 spectrometer (600.03 MHz 1H frequency) with a triple axis gradient, triply tuned (1H , ^{15}N , ^{13}C) inverse configuration probe. The probe temperature was maintained at 25 $^{\circ}C$. The pulse sequence used was a one-dimensional-Carr-Purcell-Meiboom-Gill (1D-CPMG) sequence using water presaturation (64 Hz field strength) during the recycle delay (3.3 s), an echo delay of 0.000320 s and the loop counter for the number of echos was set to 128. This experiment was used instead of the one-dimensional presaturation-Nuclear Overhauser Enhancement Spectroscopy (1D presat-NOESY) to minimize the signal observed from protein in the mouse urine. The acquisition time was 1.95 s using 32 K complex points for a 14 ppm sweep width. The number of scans collected for each urine sample was 256 and the data were processed using 0.3 Hz exponential line broadening. Spectra were manually phased and then baseline corrected before reducing the data using the AMIX software (Bruker-Biospin, Inc). Data reduction involved dividing the spectra into 438 segments, each 0.02 ppm wide for the spectral window ranging from 0.64 to 9.40 ppm. The regions from 4.72 to 4.92 (water) and 5.50 to 6.13 (urea) ppm were excluded so that 398 bins were used for the statistical analysis. The sum of the area for each segment was used to define each variable and each variable was scaled by the total intensity of the 398 bins and a scale multiplier of 100 was used. The preprocessing option used for principal component analysis (PCA) and partial least squares – discriminant analysis (PLS-DA) was autoscaling.

The reduced data described above were imported into Simca-P+ version 11.0.0.0 by Umetrics AB (Umeå, Sweden) for PCA and PLS-DA. Multivariate analysis is well documented^[15–17] and has been applied in the areas of analytical science and specifically, metabolomics.^[18,19] Briefly, PCA is a mathematical manipulation of the data whereby linear combinations of the reduced data are optimized with respect to the variance and are plotted using axes redefined by the factors or principal components instead of the original measurement variables. With the plot just described, called the scores plot, the analyst can view the multivariate nature of the data using only two or three dimensions enabling human pattern recognition to see features in the data if they exist. In the PCA, the variables responsible for the features in the scores plot can be obtained from the loadings plot. A loadings plot can be generated for each axis of the scores plot yielding a one-, two- or three-dimensional plot that indicates which variables are most important for the separation seen along each factor. In the present case, these variables represent the proton chemical shifts of metabolites and in many cases allow for their identification. PLS-DA is a multivariate analysis method similar to PCA but whereas PCA is an unbiased statistical procedure, in PLS-DA, the samples are put into classes (for example, drug-treated or untreated). PLS-DA uses a regression technique that rotates the PCA components to achieve maximal separation of these classes. The regions of the spectra that were identified as being characteristic of a class were then identified using an in-house library of reference spectra using the SBASE feature built into the AMIX software. Not all the peaks could be associated with a known

metabolite and searching the Madison Metabolomics Consortium Database,^[20] orotic acid was also identified. The other peaks have unknown identities and the supplemental material contains the lists of the spectral regions ranked for their importance in the comparison of the various classes defined in the PLS-DA (as discussed later in the text). Those peaks with unknown identities were not artifacts of the autoscaling preprocessing, that is, noise regions, as it is acknowledged that autoscaling has the potential of identifying noise regions as significant. All spectra were carefully reviewed after statistical analysis using the data-viewing tools of the AMIX software. Similar statistical results for the PCA were obtained using pareto scaling (data not presented).

Results and Discussion

Some biological endpoints from the study are presented in Table 1. The data in the table show that captopril-treated mice formed less plaque than untreated mice. This is consistent with previous literature showing that captopril reduced the aortic lesion area by 70% in apoE-deficient mice compared with untreated mice.^[10] During the course of the study, food consumption for the animals in cages 1, 2 and 4 was about the same (within 5%) averaging 3.1 g/day per animal, whereas for the mice in cage 3, the average was 2.3 g/day per animal. Mouse 14 appears mildly hypoglycemic relative to the other mice and exhibited the highest plaque formation among the captopril-treated mice. It is difficult to reconcile mouse 14, except that occasionally in these types of studies you observe outliers regardless of food/fat consumption. Captopril was in powdered form and dispersed throughout the food so mouse 14 could not avoid consuming the drug. Measurements of drug levels were not made. No unusual behavioral patterns were observed in the drug-treated group. Animals 7, 8, 11 and 15 were not transferred to metabolism cages for urine collection and so they are not part of the NMR-based urine profiling presented in the following sections. This group served as a control to show that the extra handling of the animals to collect urine did not create a bias in the results due to additional stress placed on the animals.

The upfield region of the proton NMR spectra of urine collected at the end of the study is shown in Fig. 1 for captopril-treated (top, mouse 16) and untreated mice (bottom, mouse 4). For the sake of comparison, the spectra were scaled relative to the creatinine singlet resonance at approximately 4.1 ppm. The peaks represent only the endogenous metabolites from the mouse urine as the resonances from captopril were not observed in the spectra. From a casual glance at the spectra in Fig. 1, one can see that the peaks appear very similar but there are differences in peak intensities. These differences for all the spectra will be dissected in the following sections using multivariate analysis.

PCA is a multivariate analysis technique used to visualize trends within complex sets of data and it enables the analyst to then query the data to find those variables that are responsible for the trends. Figure 2 shows the scores plot using the first two principal components. Each point represents the urine sample from a specific mouse at a particular collection time. The filled triangles are for the captopril-treated mice and the crosses represent the untreated mice. The urine samples were collected every 2 weeks and the labels indicate the time point in the study beginning at week 2 (2w) and continuing to week 14 (14w). The first two principal components (out of a total of 11) contain 40% of the explained variation and 34%

of the predicted variation. What is readily seen is that the two groups overlap at the beginning of the study and they diverge toward the end of the study. Since at the first time point (2 weeks), the drug-treated and untreated mice appear juxtaposed, it would suggest that captopril is well tolerated. There was an exception in the captopril-treated group, all of the data points – from start to finish – for animal 16 are in the upper left quadrant. Focusing on the untreated animals, their data points start in the region of the upper right quadrant and migrate toward the lower left quadrant, whereas the captopril-treated group trend with time toward the upper left quadrant. The captopril-treated data points are also more diffuse. The divergence of the two treatment groups appears greater if the data are viewed in three dimensions using the first three principal component axes.

The trend with time could possibly be explained as the effect of aging and the separation of the two groups as the effect of drug treatment and/or amelioration of the disease condition. From the loadings found from the PCA, the captopril-treated group did show an increase in allantoin relative to the control group. In mice, allantoin is enzymatically formed from uric acid as it is more soluble than uric acid. An increase in allantoin is consistent with the observation of mild uricosuria in humans treated with captopril.^[21] Humans do not have the enzyme to convert uric acid to allantoin. The apoE knockout phenotype will exhibit the formation of plaques in 3–4 months time on a high-fat diet, thus movement away from the starting point is expected whether it be due to aging or disease progression. The life expectancy of the mice is only about 2 years. We have observed such a migration over time using rats (unpublished observation) and this type of shift of metabolite patterns with time has been reported in the literature.^[22] The fact that clustering was observed means that the data will be amenable to further statistical analysis to better differentiate the groups and seek characteristic metabolite markers.

For classification of the differences, PLS-DA was applied to the 2-week and 14-week data points excluding animal 16 from the 2-week group and the result of this is shown in Fig. 3. For the modeling, three classes were created: one class included the vehicle- and captopril-treated animals at 2 weeks, the next class was the untreated animals at 14 weeks and the third class was the captopril-treated animals at 14 weeks. The vehicle- and captopril-treated animals at 2 weeks were put in one class because ANOVA of the PCA scores showed that there was not a statistically significant difference between these two groups of animals (the F ratio was much less than the F_{critical} value). Two principal components were generated to classify the groups. The validation of the PLS-DA models using a regression plot of permutations gave Q^2 values less than 0.05 and R^2Y values of 0.24 that are satisfactory values for these parameters.^[16] The three groups separate well with the 2-week animals in the right half of the scatter plot, the 14-week vehicle group in the upper left quadrant and the 14-week captopril-treated group in the lower left quadrant except for animal 14 that is seen in the upper left quadrant toward the center of the plot. At 14 weeks, animals 2, 4, 13 and 14 are in a region that could be considered intermediate as they have not advanced as far as their counterparts in the upper left (untreated) or lower left (captopril-treated). This is supported by the contributions plot that shows only moderate differences between this group and the group at 2 weeks when using two standard deviations as the threshold (data not shown).

The animals that we focused on to best characterize the groups were animals 1, 3, 5, and 6 for the 14-week untreated group; animals 9, 10, 12, and 16 for the 14-week captopril-treated group; and all of the animals for the 2-week group. The comparison of the drug-treated and untreated mice at the end of the study (14 weeks) from PLS-DA can readily be viewed from the contributions plot (Fig.

4). The contributions plot shows those regions of the spectrum that are most important for the classification of each group. The positive bars indicate those regions of the spectrum that are increased for the vehicle group and the negative bars for those spectral regions increased for the 14-week captopril group. We focused on those regions that had a difference greater than two standard deviations. Table 2 shows the metabolites that could be identified that characterized the differences among the three groups. Those buckets not identified can be found in the complete listings (x,y pairs) of the contributions plots comparing the different classes in the supplemental information. In the captopril-treated group, allantoin is elevated as noted earlier and also *N*-methylnicotinamide (NMN). The NMN is of interest because it was suggested to be a marker for peroxisome proliferation^[23] and for type 2 diabetes.^[24] In the type 2 diabetes reference, there was a concomitant decrease in allantoin that is related to NMN because it is a breakdown product of nucleotide metabolism. Allantoin is on the degradation pathway of adenine and NMN is a product of nicotinamide *N*-methyltransferase (EC 2.1.1.1) in the pathway of nicotinate and nicotinamide metabolism. NMN was reported as having antiinflammatory properties.^[25,26] Of particular interest to this report is the proteomic and metabolomic study of apoE-deficient mice where they reported some metabolite markers of oxidative stress in aorta tissue.^[27] They reported a reduction in the adenosine nucleotide pool in young apoE knockout mice and an increase in xanthine and hypoxanthine that are degradation products of adenosine. The authors note that these metabolites are utilized by xanthine oxidase that is a powerful enzyme in the generation of reactive oxygen species (ROS).^[27,28] In the present study, xanthine was elevated in the contributions plot for the untreated mice relative to the captopril-treated mice at 14 weeks. Allantoin is downstream in the biochemical pathway from xanthine (and hypoxanthine) but allantoin levels were higher in the captopril-treated mice. This might be explained by captopril's effect on the kidneys that could be facilitating the excretion of allantoin.^[21] Higher levels of ascorbate were observed in the untreated group relative to the captopril-treated group. Mice have the enzyme, *L*-gulonolactone oxidase that enables them to synthesize ascorbate (vitamin C).^[29] A mammalian feedback mechanism increases the daily ascorbate production many fold under stress.^[30,31] Possibly the untreated mice in response to the plaque formation have increased their production of vitamin C. The plaque formation, although not occluding the artery, may be perceived as a stress and there is some literature precedence for animals to greatly increase their vitamin C output in response to stress or possibly its role is to improve vasodilation.^[32]

The comparison of the captopril-treated mice *versus* the group at the beginning of the study highlights effects that could be attributed to the captopril. Support for this may be the elevated indoxyl sulfate in the captopril-treated mice. Indoxyl sulfate is known to be a uremic toxin stimulating glomerular sclerosis and interstitial fibrosis.^[33,34] The decrease in the ascorbate in the captopril-treated group could be related to lower plaque load as seen in Table 1 for the captopril-treated mice reflecting lowered levels of vascular stress. This

observation is also seen in the comparison of the drug-treated and untreated mice at 14 weeks where the untreated mice have higher ascorbate levels.

The comparison of the untreated mice at 14 weeks to the 2-week group shows that xanthine is elevated at 14 week that is a breakdown product of purines and orotic acid is elevated, an intermediate in the synthesis of pyrimidine nucleotides. Trimethylamine was also elevated in the 14-week untreated group relative to the control group. Trimethylamine is generated by gut microflora from the catabolism of dietary choline.^[24] The results observed from the urine samples would not be expected to have markers of plaque formation that would be found in plasma, such as elevated cholesterol. The interesting metabolite in this comparison is xanthine because it has been reported as a marker of inflammation associated with plaque formation.^[27] In our study, treatment with captopril reduced this metabolite and possibly is responsible for the lower levels of vitamin C observed in the drug-treated group since ROS have conceivably been lowered. (Xanthine is formed from xanthine oxidase that is known to generate ROS.) In this comparison, those bins above the threshold of two standard deviations could not be identified for the group at 2 weeks and thus that region of the table is left blank (middle right section of Table 2).

Returning to Table 1, it is desirable to consider the outliers. For example, mouse 2 had the highest percentage of plaque in the untreated group and mouse 4 had the lowest percentage of plaque and in the captopril-treated group, mouse 14 had plaque formation similar to the untreated animals. Comparison of the position of mice 2 and 4 on the scores plot (Fig. 3) has them right next to each other near the center of the plot but interestingly mouse 14 was found in the quadrant containing the untreated mice. If the focus is drawn to the spectra to compare the ascorbate and xanthine peaks, no correlation was found for the outliers between the percentage of plaque and the peak intensities of the xanthine and ascorbate peaks. There was of course a trend for the metabolites between the untreated and captopril-treated mice as shown using the PLS-DA. This leads to the following question: what is the significance between the statistical results based on the NMR spectra of the urine and the amount of plaque formed. The results have reflected an effect of the drug that we have ascribed to the elevated allantoin in the drug-treated group, and based on our findings and in agreement with another study we have proposed the xanthine level in the untreated group as a marker for the plaque formation.^[27] The elevated ascorbate in the untreated group is consistent with findings that stress will cause more ascorbate to be produced, be it to counter greater ROS or for vasodilation. There are possibly more features in the data, due to our inability to assign metabolite identities to all the resonances, that might strengthen or weaken the connection between the separation of the groups observed in the PLS-DA and the biological endpoint – arterial plaque formation.

Since the PCA scores plot showed a trend as a function of time, we chose to pick the time points at the end of the study where the separation of the samples was the greatest to analyze the differences. It is worth pointing out, however, that some of the metabolites which were found to differentiate the groups were seen in the middle time points. For example, ascorbic acid and xanthine were seen at higher levels in the untreated mice than in the captopril-treated mice. These metabolites are of particular interest because of their role regarding oxidative stress as discussed previously. Also, *N,N*-dimethylglycine and orotic acid levels

were elevated in the untreated mice at earlier time points. These are all metabolites that appeared in the animals that had greater plaque formation.

In conclusion, we believe that our evaluation of NMR-based metabolomics of mouse urine in the apoE-deficient mouse model of atherosclerosis yielded positive results. Metabolites were observed that we believe were indicative of disease progression because of their role in oxidative stress. There were also other metabolites found whose roles in the disease progression were unknown (see Table 2 and preceding discussion). Allantoin, we ascribed as a marker for the drug treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Assmann G, Carmena R, Cullen P, Fruchart JC, Jossa F, Lewis B, Mancini M, Paoletti R. *Circulation*. 1999; 100:1930. [PubMed: 10545439]
2. Brown MS, Goldstein JL. *Science*. 2006; 311:1721. [PubMed: 16556829]
3. Paigen B, Morrow A, Holmes PA, Mitchell D, Williams RA. *Atherosclerosis*. 1987; 68:231. [PubMed: 3426656]
4. Breslow JL. *Science*. 1996; 272:685. [PubMed: 8614828]
5. de Winther MP, Hofker MH. *Curr. Opin. Lipidol.* 2002; 13:191. [PubMed: 11891422]
6. Paigen B, Plump AS, Rubin EM. *Curr. Opin. Lipidol.* 1994; 5:258. [PubMed: 7981956]
7. Svenson KL, Bogue MA, Peters LL. *J. Appl. Physiol.* 2003; 94:1650. discussion 1673. [PubMed: 12626479]
8. Nakashima Y, Plump AS, Raines EW, Breslow JL, Ross R. *Arterioscler. Thromb.* 1994; 14:133. [PubMed: 8274468]
9. Plump AS, Smith JD, Hayek T, Aalto-Setälä K, Walsh A, Verstuyft JG, Rubin EM, Breslow JL. *Cell*. 1992; 71:343. [PubMed: 1423598]
10. Hayek T, Attias J, Smith J, Breslow JL, Keidar S. *J. Cardiovasc. Pharmacol.* 1998; 31:540. [PubMed: 9554802]
11. Daugherty A, Rateri DL. *Methods*. 2005; 36:129. [PubMed: 15893934]
12. Daugherty A, Whitman SC. *Methods Mol. Biol.* 2003; 209:293. [PubMed: 12357958]
13. Palinski W, Ord VA, Plump AS, Breslow JL, Steinberg D, Witztum JL. *Arterioscler. Thromb.* 1994; 14:605. [PubMed: 7511933]
14. Tangirala RK, Rubin EM, Palinski W. *Lipid Res J.* 1995; 36:2320.
15. Beebe, KR.; Pell, RJ.; Seasholtz, MB. *Chemometrics: A Practical Guide*. John Wiley and Sons; New York: 1998.
16. Eriksson, L.; Johansson, E.; Kettaneh-Wold, N.; Wold, S. *Multi- and Megavariate Data Analysis, Principles and Applications*. Umetrics AB; Umea: 2001.
17. Jackson, JE. *A User's Guide to Principal Components*. John Wiley and Sons; New York: 1991.
18. Eriksson L, Antti H, Gottfries J, Holmes E, Johansson E, Lindgren F, Long I, Lundstedt T, Trygg J, Wold S. *Anal. Bioanal. Chem.* 2004; 380:419. [PubMed: 15448969]
19. Holmes E, Shockcor JP. *Curr. Opin. Drug Discov. Dev.* 2000; 3:72.
20. Cui Q, Lewis IA, Hegeman AD, Anderson ME, Li J, Schulte CF, Westler WM, Eghbalian HR, Sussman MR, Markley JL. *Nat. Biotechnol.* 2008; 26:162. [PubMed: 18259166]

21. Reyes AJ. *Cardiovasc. Drugs Ther.* 2003; 17:397. [PubMed: 15107595]
22. Bollard ME, Holmes E, Lindon JC, Mitchell SC, Branstetter D, Zhang W, Nicholson JK. *Anal. Biochem.* 2001; 295:194. [PubMed: 11488622]
23. Delaney J, Hodson MP, Thakkar H, Conner SC, Sweatman BC, Kenny SP, McGill PJ, Holder JC, Hutton KA, Haseldon JN, Waterfield CJ. *Arch. Toxicol.* 2005; 79:208. [PubMed: 15838709]
24. Salek RM, Maguire ML, Bentley E, Rubtsov DV, Hough T, Cheeseman M, Nunez D, Sweatman BC, Haselden JN, Cox RD, Connor SC, Griffin JL. *Physiol. Genomics.* 2007; 29:99. [PubMed: 17190852]
25. Gebicki J, Sysa-Jedrzejowska A, Adamus J, Wozniacaka A, Rybak M, Zielonka J. *Pol. J. Pharmacol.* 2003; 55:109. [PubMed: 12856834]
26. Gosteli J. *Med. Hypotheses.* 2005; 64:1062. [PubMed: 15780513]
27. Mayr M, Chung Y-L, Mayr U, Yin X, Ly L, Troy H, Fredericks S, Hu Y, Griffiths JR, Xu Q. *Arterioscler. Thromb. Vasc. Biol.* 2005; 25:2135. [PubMed: 16123314]
28. Berry CE, Hare JM. *Physiol J.* 2004; 555:589.
29. Chatterjee IB. *Science.* 1973; 182:1271. [PubMed: 4752221]
30. Subramanian N, Nandi BK, Majumder AK, Chatterjee IB. *Biochem. Pharmacol.* 1973; 22:1671. [PubMed: 4147115]
31. Subramanian N. *Inflamm. Res.* 1978; 8:484.
32. Padayatty SJ, Levine M. *Am. J. Clin. Nutr.* 2000; 71:1027. [PubMed: 10799361]
33. Aoyama I, Miyazaki T, Niwa T. *Miner. Electrolyte Metab.* 1999; 25:365. [PubMed: 10681668]
34. Niwa T, Aoyama I, Takayama F, Tsukushi S, Miyazaki T, Owada A, Shiigai T. *Miner. Electrolyte Metab.* 1999; 25:118. [PubMed: 10207273]

Figure 1.

The upfield region of the 600 MHz proton NMR spectra of the urine collected at week 14 of the study for the captoril-treated (top, mouse 16) and untreated mice (bottom, mouse 4). The spectra were scaled relative to the creatinine singlet resonance at 4.06 ppm.

Figure 2.

The principal component analysis scores plot using the first two principal components showing all the data points from the study. The filled triangles are for the captopril-treated mice and the crosses represent the untreated mice. The labels indicate the time points in the study and the samples were collected beginning at week 2 (2w) and continuing to week 14 (14w).

Figure 3. Partial least squares–discriminant analysis scores plot generated from the 2-week and 14-week data points excluding animal 16 from the 2-week group. Three classes were created: The squares are the vehicle- and captopril-treated animals at 2 weeks, the crosses are the untreated animals at 14 weeks and the triangles are the captopril-treated animals at 14 weeks. The labels correspond to the mouse identifications used in Table 1.

Figure 4.

The partial least squares–discriminant analysis contributions plot comparing the drug-treated and untreated mice at the end of the study (14 weeks). Positive bars indicate the vehicle group and negative bars are for the captopril group. The y-axis is in units of standard deviations. The x-axis represents the bin labels and is functionally the proton chemical shift scale in ppm.

Table 1

Biological measurements recorded at the end of the study

Mouse id	Cage/Group	Final weight (g)	Glucose (mg/dl)	% Plaques
1	1/Untreated	30.3	172	2.5
2	1/Untreated	29.2	254	8.0
3	1/Untreated	30.3	171	4.1
4	1/Untreated	32.7	145	1.6
5	2/Untreated	33.9	196	3.9
6	2/Untreated	34.3	185	5.6
7	2/Untreated	31.7	188	2.6
8	2/Untreated	39.7	219	2.5
	Mean	32.8	191.3	3.9
	SD	3.3	33.17	2.1
	SE	1.2	11.73	0.7
9	3/Captopril	29.4	219	1.4
10	3/Captopril	25.7	243	0.1
11	3/Captopril	23.1	158	0.3
12	3/Captopril	23.4	219	0.0
13	4/Captopril	34.4	230	0.8
14	4/Captopril	29.0	117	4.5
15	4/Captopril	29.4	180	0.0
16	4/Captopril	30.3	180	1.4
	Mean	28.1	193.3	1.1
	SD	3.8	42.36	1.5
	SE	1.3	14.98	0.5

Table 2

Metabolites identified from the partial least squares–discriminant analysis contributions plots

Captopril-treated mice – 14 week	Control group – 2 week
Allantoin	Ascorbic acid
Dimethylamine	<i>N,N</i> -dimethylglycine
Indoxylsulfate	Urocanic acid
1- <i>N</i> -methylnicotinamide	
Trimethylamine	
Untreated mice – 14 week	
Orotic acid	
Trimethylamine	
Xanthine	
Captopril-treated mice – 14 week	Untreated mice – 14 week
Allantoin	Ascorbic acid
1- <i>N</i> -methylnicotinamide	Orotic acid
	Urocanic acid
	Xanthine