Free Radical Research, Volume 37 Number 11 (November 2003), pp. 1209–1213

Taylor & Francis
healthsciences

Hydrogen Peroxide in Urine as a Potential Biomarker of Whole Body Oxidative Stress

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Accepted by Dr H. Sies

(Received 24 July 2003; In revised form 2 August 2003)

The level of hydrogen peroxide (H_2O_2) in urine has been suggested as a potential biomarker of whole body oxidative stress, but issues of stability, reproducibility and biological variation have not been investigated to date. In this study, we used a refined protocol, which demonstrated improved sensitivity and precision, to determine the stability of H_2O_2 in urine, and to measure its concentration in apparently healthy subjects. We also investigated intra-individual variation within and between days. Results showed that H_2O_2 in urine is stable for up to 48 h at 4°C, however, storage of urine at room temperature was associated with up to 50% increase in H_2O_2 concentration over a few hours. Total H_2O_2 in freshly voided urine from 55 healthy, fasting subjects ranged from 0.84 to 5.71 μM, or 90–1164 μmol H_2O_2/mol creatinine. Intra-individual variation was wide. Even when concentration corrected and collected at the same time of day, 2- to 3-fold variation was seen over 4 consecutive days, and over the course of a single day the creatinine-corrected H_2O_2 also varied significantly. We suggest that this large biological variation limits the usefulness of urine H_2O_2 as a biomarker of oxidative stress, the exception being when the effects of disease, therapy or diet induce very large changes in its concentration.

Keywords: Hydrogen peroxide; Urine; Oxidative stress; ROS

INTRODUCTION

There are various biological markers that are used to assess oxidative stress, including F2 isoprostanes, 8-oxo-dG, and protein carbonyls. These biomarkers give a view of oxidative damage to, respectively, lipid, DNA and protein, but individually do not give a "global" view of whole body stress.[1–5] Furthermore, these tests are expensive and difficult to perform well. A simple and inexpensive test of overall oxidative stress would be a useful addition to the test menu for researchers investigating oxidative stress and diet in relation to ageing and chronic degenerative disease. In this regard, it has been suggested that the level of hydrogen peroxide (H_2O_2) in urine may be a possible candidate for a biomarker of the extent of whole body oxidative stress.[6–8] This is an attractive concept, as urine is easy to obtain, and H_2O_2 is simple to measure.

Before assessing the effect of dietary change, lifestyle, ageing or disease on a putative biomarker, however, its biological, or "normal", variation must be determined within- and between individuals, and the stability of the biomarker in samples *ex vivo* must be determined.[9] Our primary aims in this study, therefore, were to investigate inter- and intra-individual variation in urine H_2O_2 concentration in healthy subjects and to determine the effect of urine storage at room temperature and at 4°C. We employed a commonly used, technically simple method for H_2O_2 measurement, often referred to as the FOX assay,[8–12] which employs ferrous ion oxidation by hydrogen peroxide, with subsequent formation of a blue-violet coloured Fe^{2+}-xylenol orange chromogen. Because the assay showed rather poor precision and sensitivity in our preliminary studies, we aimed also to refine the method to lower the limit of detection and increase the precision.

MATERIALS AND METHODS

All chemicals and reagents were of analytical grade. To prepare the working FOX reagent,

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2003 Taylor & Francis Ltd
DOI: 10.1080/1071576031000161603 2

1 ml of a 25 mM aqueous solution of ferrous ammonium sulphate $FeSO_4(NH_4)_2SO_4{\cdot}6H_2O$ (Sigma, St. Louis, MO, USA) in 2.5 M H_2SO_4 (Merck Chemical Co. Ltd, Darmstadt, Germany) was mixed with 100 ml of an aqueous solution of 125 μM xylenol orange (3,3-bis[*N*,*N*-Di(carboxymethyl)aminomethyl]-*o*-cresol-sulphonaphthalein; Sigma) containing 100 mM Sorbitol (>99% pure, BDH Laboratory Supplies, Poole, England). This solution was prepared just before use. A 30% H_2O_2 stock solution (Sigma) was used to prepare working H_2O_2 standards over the range 0–15 μM. Working standards (0, 2, 4, 6, 8, 10, 12 and 15 μM) were prepared freshly as needed from the stock 30% solution and using MilliQ ultrapure water (at least 18.2 MΩ, prepared using a Millipore Ultrapure Water System, Millipore Corp., Bedford, MA). The working standards, controls and urine samples were treated identically using two protocols. One protocol followed the published FOX assay, as used in a commercially available kit method (Pierce Chemical Company, Rockford, USA) as follows: 20 μl of standards, control or urine was added to 200 μl of reagent (freshly prepared as described above) in a microplate well, mixed and left for 20 min at room temperature, and the absorbance at 590 nm was read against a reagent blank. The other protocol was based on this, however, in an attempt to improve the sensitivity of the assay we increased the sample : -working reagent ratio, adding 100 μl sample to 170 μl reagent in a microplate well. Furthermore, because we noted that colour development had not stabilised after the 20 min reaction time recommended, we extended the incubation time after sample/reagent mixing to 60 min, by which time colour development was approaching a plateau value. Both protocols were performed in parallel at room temperature, using flat bottomed microtitre plate wells (Thermo LabSystems, Franklin, MA) and the same standards, controls and urine samples. Absorbance readings were taken using a TECAN SPECTRAFluor Plus microplate reader (TECAN Austria GmbH, Grodig, Austria). The limit of detection for each protocol was determined by calculating the mean + 3SD absorbance value of a blank (MilliQ water) measured 20 times by each protocol, and reading the corresponding value from the appropriate calibration line. Precision, in terms of in-run and between-run coefficients of variation (CV), was determined using freshly prepared solutions of H_2O_2. All urine samples were performed in duplicate by each protocol, and the mean value used.

To investigate inter- and intra- individual variation in urine H_2O_2, mid-stream urine samples were collected, into plain containers, from apparently healthy volunteers aged 20–55 years. Fasting urines from 55 volunteers (29 men, 26 women) were collected between 9 and 10 a.m. to investigate inter-individual variation. In a separate experiment, 17 volunteers provided mid-stream urine samples at 9 a.m. (fasting), 12 midday, 2 p.m. and 4 p.m. on the same day. In a third experiment, 15 volunteers provided a mid-stream urine sample on each of 4 consecutive days: the time at which the urine was collected was the same for each subject on each of the 4 days, however, nine volunteers collected urine each morning at 10 a.m., while six collected urine at 2 p.m. each day. Freshly voided urines were immediately centrifuged at 4°C for 8 min at 2500 rpm, and H_2O_2 measurements were performed immediately thereafter, using the refined protocol. Aliquots of centrifuged fasting urines were stored at 4°C for evaluating the effect of up to 48 h storage. In a separate study, the effect of up to 6 h storage at room temperature compared to 4°C was also investigated.

H_2O_2 results were concentration corrected and expressed as μmol H_2O_2 per mol creatinine, as well as in μM total H_2O_2. Creatinine in urine (diluted in water as appropriate) was measured by an automated commercial creatinine assay kit (Biosystems Reagents and Instruments, Barcelona, Spain), which is based on the method of Jaffe.[13]

Ethical approval for the study was obtained from the University Ethics Sub-Committee, and all procedures involving human subjects complied with the Declaration of Helsinki as revised in 2000.

Statistical analysis: GraphPad Prism version 3.0 for Windows (GraphPad Software, San Diego, CA) was used to perform the Friedman test with Dunnett's post test for intra-individual within-day and between-day variation. The paired *t*-test was used to assess *ex vivo* changes. Statistical significance was sought at the 5% level.

RESULTS

After increasing the sample : reagent ratio and extending the incubation time from 20 to 60 min, the limit of detection was around 3-fold lower, and precision and sensitivity were markedly improved. In-run CVs for the original and refined protocols were, respectively, 3.37 and 1.99% (at 5.0 μM H_2O_2; $n = 11$ in each case). Between-run CVs were, respectively, 4.98 and 2.17% at a similar H_2O_2 concentration ($n = 9$ in each case). The limits of detection of H_2O_2 with the original and refined protocols were, respectively, 2.5 and 0.8 μM. Figure 1 shows the dose-response curves obtained with the original protocol (squares) and with the refined protocol (circles). It can be seen that the sensitivity (gradient of the dose-response line) is almost 4-fold

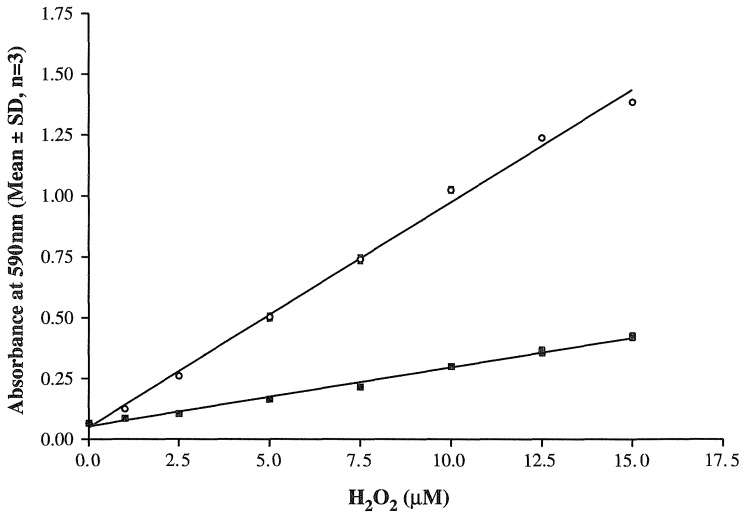

FIGURE 1 Dose-response (calibration) lines for the original (squares) and refined (circles) protocols for H_2O_2 measurement.

better using the refined protocol. Linearity was maintained at least to 12.5 μM H_2O_2.

In regard to *ex vivo* changes, urine stored at 4°C for up to 48 h showed no significant changes ($P > 0.05$) in H_2O_2 concentration: Mean (SD) at zero time, +5, +24 and +48 h were, respectively, 2.57 (1.19), 2.74 (1.48), 2.70 (1.38) and 2.63 (1.48) μM ($n = 55$ in each case). Results of six urine samples that were stored and measured, in parallel, at room temperature and 4°C showed that with storage at room temperature, urine H_2O_2 increased by around 40% over 2 h, and by 50% by 4 h, but again no appreciable difference was seen in urines stored at 4°C (results not shown).

In regard to biological variation, total and creatinine-corrected H_2O_2 results on fasting urine samples ($n = 55$) are shown in Table I. Urine total H_2O_2 concentrations ranged from 0.84 to 5.71 μM; concentration-corrected levels ranged from 90 to 1164 μmol H_2O_2/mol creatinine. There was no significant difference between urine H_2O_2 concentrations in men and women. There was a significant correlation between H_2O_2 concentration and creatinine concentration ($r = 0.531$; $P < 0.0001$), however, scatter was wide, indicating that differences in H_2O_2 concentration were not a simple function of urine concentration *per se*. Within-day intra-individual variation is shown in Fig. 2. From these data it can be seen that the creatinine-corrected H_2O_2 concentration increased significantly ($P < 0.05$) during the day in both men and women, when compared to the fasting (9 a.m.) sample, with highest values seen in samples collected in the early afternoon (2 p.m.). Individual, between- day variation in 15 subjects is shown in Fig. 3. Results indicate that intra-individual variation in some subjects was very wide.

TABLE I Urine H_2O_2 concentrations in healthy, fasting adults (Mean; Median (SD); $n = 55$)

	Total H_2O_2 (μM)	Concentration corrected (μmol H_2O_2/mol creatinine)
All ($n = 55$)	2.57; 2.34 (1.19)	322; 229 (233)
Men ($n = 29$)	2.71; 2.34 (1.17)	346; 265 (266)
Women ($n = 26$)	2.42; 2.34 (1.22)	301; 214 (200)

DISCUSSION

The use of biomarkers indicating oxidative stress is an important issue.[9] In this study we used a refined protocol, which demonstrated improved sensitivity and precision, to determine the stability of H_2O_2 in urine, and the concentration in fasting urine of 55 apparently healthy subjects in order to investigate whether the level of H_2O_2 in urine is a suitable potential biomarker for use in studies of oxidative stress and the effect of age, disease, and diet on this. We also investigated the effect of sampling time and between-day intra-individual variation. Results showed that H_2O_2 in urine is stable for up to 48 h at 4°C, however, storage of urine at room temperature was associated with up to 50% increases in H_2O_2 concentration over a few hours. This increase is presumably owing to autoxidation of urine constituents. This increase was seen also in a study by Lee *et al.* who showed increases of 17–139% in seven urine samples stored for 3.5 h at room temperature.[11]

In this current study, the concentrations of total H_2O_2 in freshly voided urine in 55 healthy subjects were lower than those previously reported.[7–8,11,14] The study by Varma and Devamanoharan,[14]

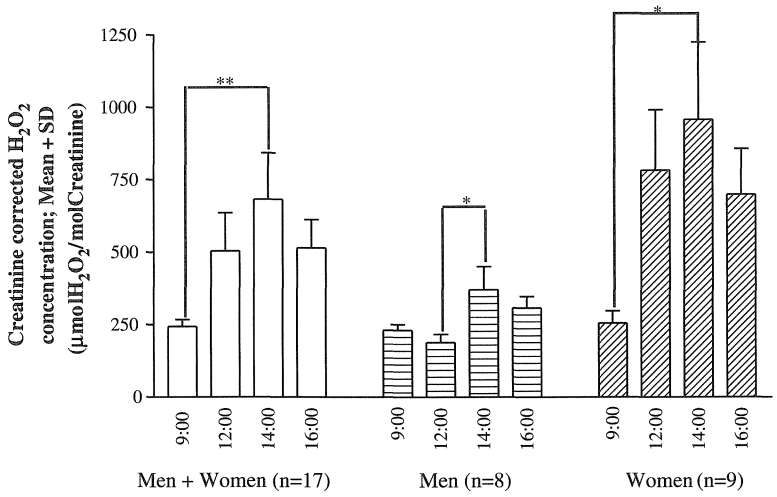

FIGURE 2 Creatinine-corrected H_2O_2 concentrations in urine of 17 healthy subjects (eight men [horizontal bars] and nine women [diagonal bars]); concentrations increased significantly ($^*P < 0.05$; $^{**}P < 0.01$) during the day.

which used a different method of measurement, reported mean (SD) urine total H_2O_2 in men and women of, respectively, 106(64) and 89(54) μM, and the studies by Lee, Halliwell and co-workers reported total levels of 0.4–109 μM in 20 healthy subjects.[7–8] In this current study, we collected urine from 55 fasting subjects, and no sample had a total H_2O_2 concentration above 6 μM. Furthermore, values in men and women were not significantly different. In terms of creatinine-corrected H_2O_2 levels in urine, recalculation of the only published data we are aware of[8] shows that spot urine concentrations in healthy, non-fasting subjects ranged from 276 to 1844 μmol/mol ($n = 4$). These values are similar to those found in this current, larger study (90–1164 μmol/mol), and again indicate a very wide inter-individual variation. Intra-individual variation in urine H_2O_2 concentrations is also large, as shown clearly in this study. Even when concentration-corrected and collected at the same time of day, 2- to 3-fold variation was seen over 4 consecutive days, and over the course of a single day, the creatinine-corrected H_2O_2 varied significantly. This was most likely owing to intake of food containing components that generate H_2O_2, but may have been related also to physical activity and electrolyte balance.[15] There is a report that drinking coffee, a beverage that has been shown[10] to generate significant amounts of H_2O_2, increased the urine H_2O_2 within 60 min in three of the four subjects studied,[8] and exercise and salt loading are also reported to affect H_2O_2 excretion.[15]

In conclusion, these new data show that H_2O_2 can be measured simply and reproducibly in human urine by the refined protocol used, and that H_2O_2 is stable in urine at 4°C for up to 48 h. The concentration varies widely, however, both within- and between day, and this, in combination with the wide inter-individual variation seen, severely limits the usefulness of urinary H_2O_2 as a potential biomarker for whole body oxidative stress unless the effects of

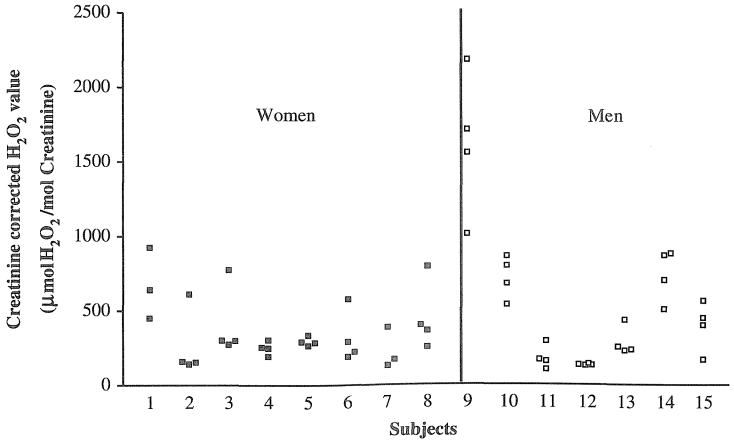

FIGURE 3 Creatinine-corrected H_2O_2 concentrations in urine of 15 healthy subjects (seven men [open squares] and eight women [closed squares]) over the course of 4 consecutive days; in each subject urine was collected at the same time each day.

disease or therapy induce very large changes in its concentration.

Acknowledgements

The authors are grateful to The Hong Kong Polytechnic University for funding this study. We thank also Dr Camus Choy for his expert technical assistance and Dr MF Chan for statistical advice.

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