ORIGINAL ARTICLE

Detection of Xanthine Oxidase In Human Plasma

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Summary

Xanthine oxidase is a highly versatile enzyme which is widely distributed among various species. Though the presence of the enzyme in serum is not yet established, high antibody titre of this enzyme has been reported. Xanthine oxidase is thought to be the principal source of free radical generation via degradation of nucleotides to the end product, uric acid. The aim of this study was to detect xanthine oxidase activity in human plasma and report any significant relationships found between its activity and variables such as race, age and sex for the sample size studied.

Forty six normal healthy individuals (14 males and 32 females) were studied. The enzyme activity was measured by a spectrophotometric method whereby the reduction of ferricytochrome c by free radicals was calculated and expressed as nmol O_2 production/ml/min.

Results obtained showed that there was a positive relationship between xanthine oxidase activity with age ($r=0.415$, $p<0.05$) and weight ($r=0.369$, $p<0.05$) in the normal individual. For the age group 30-39 yrs (n=11), a higher enzyme activity was observed in males (2.71±1.44) as compared to females (2.34±1.23) but it was not significant ($p=0.53$). For racial distribution, the Malays [M] have a higher enzyme activity (2.65±0.86; N=32) than their Indian [I] (2.27±0.58; N=7) and Chinese counterparts [C] (1.44±1.22; N=7) but this was also not statistically significant (M vs I : $p=0.39$; M vs C : $p=0.07$; I vs C : $p=0.16$).

In conclusion this study showed that there is a measurable amount of xanthine oxidase activity in the human plasma.

Key Words: Xanthine oxidase, Free radicals, Uric acid

Introduction

Xanthine oxidase is a highly versatile enzyme and widely distributed among the species. In all mammals, the liver and intestines have the highest xanthine oxidase activity[1]. Among mammals, man, pigs, sheep, cats and goats have low activities of xanthine oxidase in the serum[2]. Though xanthine oxidase is difficult to detect in the human serum, the presence of high titres of its antibody (1% to 8% of total IgG) in the serum has been reported[3].

Xanthine oxidase is recognized for its role as the rate limiting enzyme in nucleic acid degradation through which purine is channeled for terminal oxidation. Though the main physiologic role of xanthine oxidase remains unclear, there is growing evidence in the ability of this enzyme to serve as a source of oxidizing agents such as hydrogen peroxide and superoxide radicals. It is involved in the pathogenesis of ischaemic reperfusion injury to tissues such as the heart[4], kidney[5] and intestines[6]. Currently it is almost certain that free radicals in and around vascular endothelium, play a

critical role in the pathogenesis of hypertension in spontaneously hypertensive rat (SHR)[7]. Most of this free radical generation appear to be from the enzyme xanthine oxidase[8]. Using ultra-sensitive radioimmunoassay, the concentration of this enzyme was found to be 1,000 to 10,000 fold higher in the capillary endothelial cells than in other cells.

The most common method used to determine the xanthine oxidase activity is the spectrophotometric assay in which the rate of formation of oxygen free radicals from the xanthine is quantified[9]. Assays based on manometric measurement of oxygen uptake[10] is also used to measure xanthine oxidase activity. Several colorimetric methods have been described[11] as well as many radioisotopic assays[12] and fluorometric procedures[13]. A radioimmunoassay technique for detection of xanthine oxidase has recently been described[14]. In this study we performed a spectrophotometric assay using ferricytochrome c reduction by the free radical generated from xanthine (Fig. 1). The aim of this study was to detect xanthine oxidase activity in human plasma and report any significant relationships found between its activity and variables such as race, age and sex for the sample size studied.

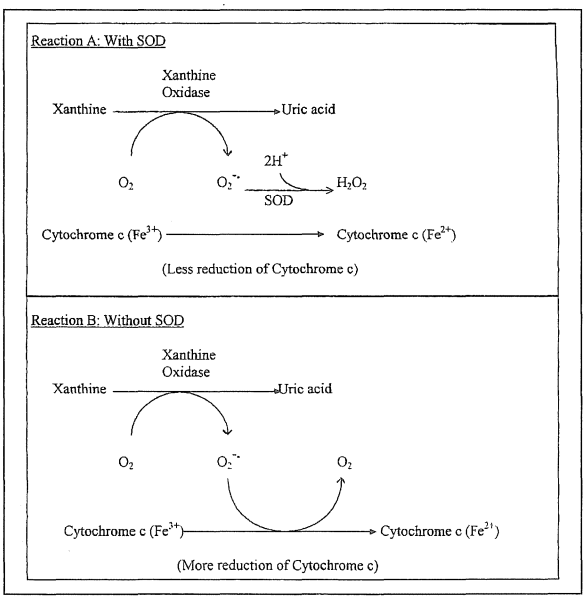

Fig. 1: Xanthine oxidase reaction in spectrophotometric assay of study. (SOD=Superoxide Dismutase)

Materials and Method

All the reagents were obtained from Sigma, USA and were of the highest available purity unless otherwise stated.

Collection of sample

Fresh blood samples were taken from 46 (Male-14, Female-32) apparently healthy normal human subjects without hypertension and gout. In vitro, xanthine dehydrogenase can be transformed into xanthine oxidase by a variety of processes. Xanthine dehydrogenase can be reversibly converted to xanthine oxidase by air oxidation[15]. Irreversible conversion of xanthine dehydrogenase to xanthine oxidase occurs through limited proteolysis by any of a number of proteins including trypsin, chymotrypsin, papain and subtilisin[9,15,16]. To minimize the reversible conversion of the enzyme, the samples were rapidly processed, that is, within two hours of sample collection. The samples were collected in processing medium containing dithiothreitol (DTT) [10mM], phenylmethylsulphonyl fluoride (PMSF) [10mM] and ethylenediaminetetraacetic acid (EDTA) [10mM]. DTT was added to the medium to reverse the conversion of xanthine dehydrogenase to xanthine oxidase 17,18 while PMSF was to act as a protease inhibitor to prevent proteolysis which can also cause artificial conversion[1].

The demographic and biological characteristics of the subjects studied are shown in Table I.

Table I
Demographic and biologic characteristics of the samples studied

Sample size (n)	46	
Race: M/I/C	32/7/7	
Sex: Male/Female	14	32
Age (y)	33.33±4.27	35.83±07.01
Weight (kg)	68.67±7.5	58.50±10.78
Height (m)	1.66±0.08	1.56±0.05

M/I/C - Malay/Indian/Chinese. Values are mean ± SD

Spectrophotometric assay of xanthine oxidase activity

The plasma was obtained by centrifugation at 1000 x g for 10 minutes at 4°C. Plasma xanthine oxidase activity was measured by adapting the method of Gerd *et al* (1989)[19] used for rat plasma. No modifications were done to the method as the assay condition per se was able to detect enzyme activity in the human plasma. In this method , xanthine oxidase activity was measured by a two-point assay using a fixed period of 10 minutes incubation at 37°C. The reaction mixture containing 200µl human plasma, 300 µL ferricytochrome c (120µM), 200 µL SOD (500 IU), and 200 µL buffer (potassium phosphate 0.0024M, sodium chloride 0.15M, pH 7.35) or 400 µL buffer in absence of SOD was pre-incubated at 37°C for three minutes. Then, 100 µL xanthine (50µM) was added to start the reaction. After 10 minutes incubation at 37°C, reduction of ferricytochrome c was measured at wavelength 550 nm in the presence/ absence of SOD.

The contents of the reference cuvette (blank) were the same except that plasma and xanthine were omitted. Absorbance of all samples were corrected for the blank. No other corrections were made for any interfering substances present in the plasma. For absorbance measurement, the same cuvette was used for all samples with or without SOD and a matched cuvette was used for the blank.

The amount of oxygen free radicals formed were calculated from the difference in the absorbance reading (±SOD). Molar extinction coefficient (ε) for cytochrome c used was 3.598×10^3 L $mmol^{-1}$ cm^{-1}. This value was the average of three absorbance readings obtained for a molar solution of cytochrome c (purity 99% from Sigma, USA). Using this ε value, concentration of xanthine oxidase was determined via the Beer-Lambert's equation, $A=\varepsilon Cl$, where A denotes maximum absorbance, ε denotes molar extinction coefficient, C denotes concentration in mM and l denotes width of the cuvette in cm[20]. Xanthine oxidase activity was expressed as nmol O_2 production/ml of plasma/ minute. A linear enzyme reaction was obtained (K_m = 16.94 µM , V_{max} = 6.33 nmol O_2/ml/min). The lowest value of xanthine oxidase detected were 0.23 nmol O_2/ml plasma/min. The intra and inter assay coefficients of variation averaged out to be 4.82%.

Statistical Analysis

All data were analyzed by Student's t-test and ANOVA (one way). Correlation and regression analysis were also done to find out the relationship of xanthine oxidase with sex, race, age, and weight. P values less than 0.05 was considered statistically significant.

Results

Correlation analysis was done between the xanthine oxidase values and the demographic variables studied. Results showed that xanthine oxidase has a strong and significant positive correlation with age (r=0.415, p=0.016) and weight (r=0.369, p=0.041) (Fig. 2). Though xanthine oxidase showed negative correlation with height (r=-0.246, p=0.117), this was not statistically significant.

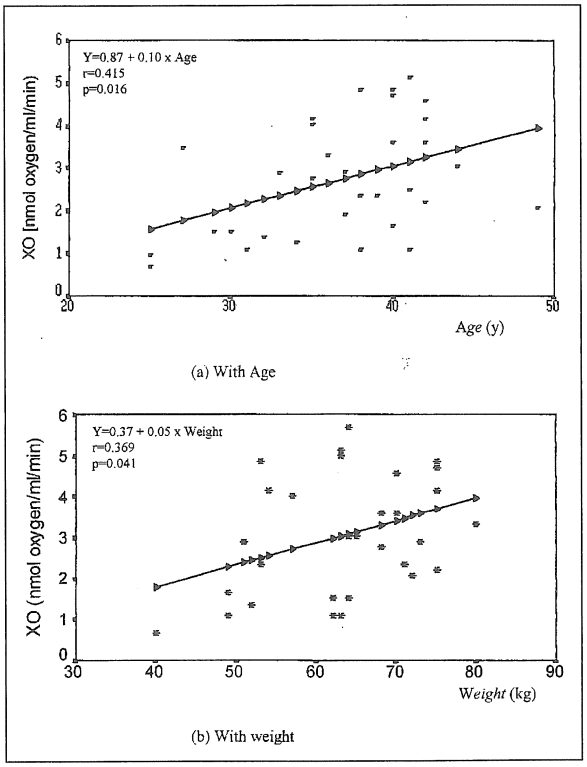

Fig. 2: Relationship of xanthine oxidase (XO) enzyme activity with demographic variables (a) with age (b) with weight

Since the enzyme activity was affected by age, valid comparison for the effect of sex can only be carried out for the age group 30-39 years with a sample size of eleven (Fig. 3). For this age group, the normal male plasma had higher xanthine oxidase activity (2.71±1.40 nmol O_2/ml plasma/min) compared to the female plasma (2.35±1.23 nmol O2/ml plasma/min) but was not statistically significant (p=0.53).

Too few samples in each ethnic group did not permit comparison of xanthine oxidase activity amongst the 3 races to be carried out for each age group. However if the subjects were matched for age, sex and weight, using paired t-test, it was found that the Malays [M] (2.65±0.86) have higher xanthine oxidase activities compared to the Indians [I] (2.27±0.58) and the Chinese [C] (1.44±1.22) but was not statistically significant (M vs I : p=0.39 ; M vs C : p=0.07 ; I vs C : p=0.16) [Fig.4].

Discussion

There are reports showing the presence of the enzyme xanthine oxidase in the liver, lung, heart, intestine and in the capillary endothelial cells[14]. Although Al-Khalidi and Chaglassian[2] reported that there was low or undetectable xanthine oxidase activity in human serum, results from our study showed the contrary. Significant xanthine oxidase activity was found in the human plasma. Presence of a measurable amount of xanthine oxidase in the human plasma in our study may be due to the shorter processing time (within 2 hrs of sample collection). We observed that after 2 hours of sample collection, xanthine oxidase activity was greatly reduced and within the next one hour, xanthine oxidase activity was no longer detectable (data not shown). Furthermore, addition of PMSF minimized the destruction of xanthine oxidase by proteases and it was reported that PMSF protection is maximum within about 2 hours of administration[21]. In our study we collected the sample directly into the processing medium composed of EDTA, PMSF and DTT to achieve maximum enzyme activity.

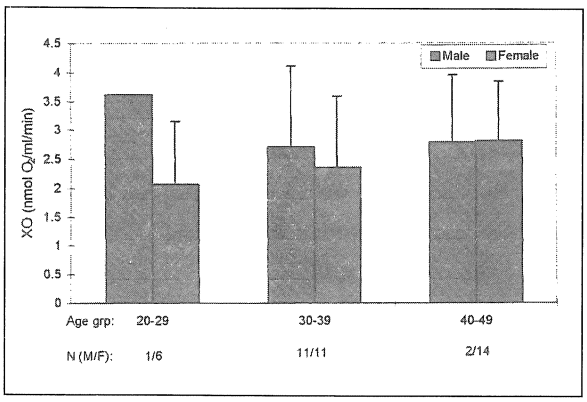

Fig. 3: Comparison of xanthine oxidase (XO) activity by sex in different age group (N= sample size, M= male, F= female)

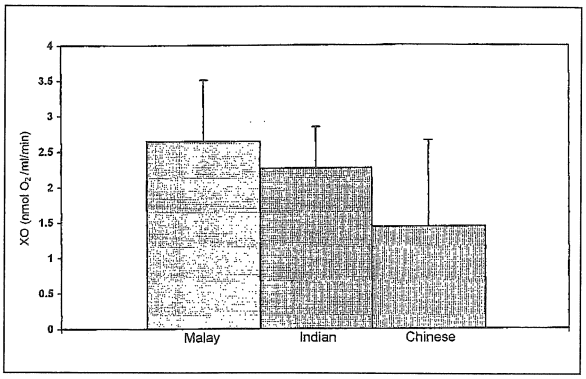

Fig. 4: Comparison of xanthine oxidase (XO) activity in different races

Al-Khalidi & Chaglassian assayed xanthine oxidase activity via uric acid production. In our study we measured the activity of the enzyme via the reduction of ferricytochrome c by free radicals (having very short half-life) produced by the enzymic reaction. Thus measurement of cytochrome c reduction seemed to be a better and more sensitive method for detection of xanthine oxidase activity in human plasma.

Even though xanthine oxidase activity was observed to be higher in Malay males, this is not statistically significant due perhaps to the small sample size. However, significant correlations were found for xanthine oxidase activity with age and weight. This implies the involvement of this enzyme in the ageing process and occurrence of degenerative diseases such as hypertension and thus cardiovascular diseases as blood vessels tend to lose their elasticity with age.

There are several studies showing the role of xanthine

oxidase in tissue reperfusion injury[4,19,22]. Xanthine oxidase is known to act on xanthine whereby uric acid and free radicals are produced. It has also been proven that the xanthine oxidase inhibitor, allopurinol, can reduce its activity[19]. Hence, it is now commonly used in the treatment of gout or hyperuricaemia. It was also reported that allopurinol can scavenge free radicals (especially OH•). At the same time oxypurinol, another potent inhibitor of xanthine oxidase, was reported to decrease blood pressure in SHR but not in normal rats[7]. If it can be proven definitely that free radicals play a role in the pathogenesis of hypertension, then, monitoring the activity of this enzyme in individuals prone to the disease will be of benefit in the prognosis of the disease.

Conclusion

From this study there is evidence that there is xanthine oxidase activity in the human plasma which has a positive relation with age and weight. Early monitoring of this enzyme in patient with gout or hyperuricaemia and possibly in patients with essential hypertension may therefore aid in the prognosis and the therapeutic effects on the disease.

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