PLASMA XANTHINE OXIDASE AND METABOLIC INDICATORS ASSOCIATION IN HEALTHY WOMEN RELATED TO AGE

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Abstract: Xanthine oxidase (XO) may generates appreciable amounts of reactive oxygen species, such as superoxide radical and hydrogen peroxide, in different pathological conditions, and also in aging. In such circumstances, XO may be released into the bloodstream, and even, to adhere to the external surface of the endothelial cells. The aim of the study is to evaluate plasma XO activity and its correlations with some metabolic indicators in apparently healthy women as a function of age. Subjects without major diseases, were included in three groups of ages: adults (30-45 years old), presenescents (50-64 years old) and senescents (65-80 years old). Plasma XO activity was significantly increased ($p < 0,001$) in the presenescent subjects compared with adult ones. A significant positive correlation was pointed out between XO and subjects age ($r = 0,4436$; $p < 0,01$). Also, a significant positive correlation was evidenced between XO and the uric acid ($r = 0,3212$; $p < 0,02$), glucose ($r = 0,2681$; $p < 0,05$), urea ($r = 0,3275$; $p < 0,01$), total cholesterol ($r = 0,3196$; $p < 0,01$), triglycerides ($r = 0,3260$; $p < 0,01$) contents and AST ($r = 0,2588$; $p < 0,01$) and ALT ($r = 0,3741$; $p < 0,01$) activities. No correlation was showed between XO and creatinine, total proteins and fibrinogen contents. Plasma XO activity increase in aging may indicate the apparent minor changes of the energy metabolism, and the enzyme role to modulate the oxidative stress in plasma and endothelial cells, and to influence the endothelium dependent vasodilatation.

INTRODUCTION

Oxidative stress plays an important role in the aging process as well as the aging accompanying diseases (Beckman and Ames, 1998, Harman, 2001; Droge, 2002). Among mechanisms involved in the generation of reactive oxygen species, xanthine oxidase is an important source of superoxide radical ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), which mediates oxidative damage of some biomolecules. In mammals xanthin oxidoreductase (XOR) is present in two interconvertible forms, xanthine dehydrogenase (XDH), which predominates “in vivo”, and xanthine oxidase (XO). XDH is characterized by high reactivity toward NAD^+, while XO has a raised reactivity toward molecular oxygen (Ichimori et al., 1999). Mammalian XDH can be converted into XO by posttranslational changes, such as the oxidation of sulfhydril groups or by limited proteolysis (McCord, 1985; Nishino and Nishino, 1997; Kayali et al., 2001). XOR has a wide specificity for reducing substrates, but its conventionally accepted role is in purine catabolism, where it catalyses oxidation of hypoxanthine to xanthine, and xanthine to uric acid (Godberget al., 2000; Doel et al, 2001; Harrison, 2002). Urate has been proposed as a major antioxidant in plasma (Harrison, 2002), but in some circumstances could be a risks factor for cardiovascular diseases (Hare and Johnson, 2003; Bergamini et al., 2009). Xanthine oxidase has been found in large amounts in the liver, kidney and intestine and in smaller amounts in the cardiac, smooth and skeletal muscles, endothelial cells and plasma. Under certain pathological conditions (hypoxia, ischemia, and especially reperfusion/reoxygenation), tissues xanthine oxidase activity significantly increases, as well as $O_2^{\bullet -}$ and H_2O_2 formation. Sometimes XO can be released from tissues into blood and adhere to the external surface of vascular endothelium influencing vascular dilatation. (Desco et al., 2002, Landmesser et al., 2002). Contradictory data are concerning the role of the endothelial bound or endothelial vascular XO in managing vascular oxidative stress and endothelial dependent vascular dilatation (Escurza et al., 2006; Nevaz et al., 2006). Little information is about plasma XO in aging (Singh et al, 2009). Xanthine oxidase reduces nitrites and nitrates and produces nitric oxide (NO) at lower oxygen pressures and under lowering pH, for instance in ischemia, hypoxia (Millar et al., 1998; Ichimori et al., 1999; Godberg et al., 2000; Doel et al., 2001). The ability of xanthine oxidase to generate $O_2^{\bullet -}$, H_2O_2 and NO has led to growing interest in further study for clarification the role of this enzyme (Verdejo et al., 2008; Wolin, 2009).

The aim of the study is to evaluate plasma XO activity and its correlations with some metabolic parameters in order to point out the relationship between the energy metabolism changes and enzyme activity in aging process.

MATERIALS AND METHODS

Human subjects, apparently healthy women were enrolled in age-groups as follows: adults (30-45 years old); presenescents (50-64 years old) and senescents (65 – 80 years old). Subjects’ selection was carried out according to the SEINEUR protocol (apparently healthy subjects, without major diseases in line with clinical, hematological,

immunological and biochemical criteria of the protocol). Exclusion criteria include: infections with viruses, bacteria, fungi, parasites, acute or chronic inflammation, malignant conditions, heart failure in the course of treatment, hypertension, diabetes mellitus, dementia, alcoholism, malnutrition, treatment with immunosupressive, anti-inflammatory drugs and hormones.

Metabolic parameters, like serum glucose, urea, creatinine, uric acid, total cholesterol, triglyceride, total proteins, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALKP) were undertaken with an Olympus AU400 autoanalyzer. Fibrinogen was assessed using a semiautomatic coagulometer Thrombotimer 4.

Plasma xanthine oxidase activity (EDTA as anticoagulant) was determined based on the nitroblue tetrazolium (NBT) reduction reaction rate measured at 540 nm (Friend and Friend, 1974; Tarpey and Fridovich, 2001). The reaction mixture contained 50mM sodium pyrophosphate buffer, pH 8.3; 50 µmoles hypoxanthine; 0.1 mM EDTA; 6.6 µg phenazinmetosulphate (PMS); 0.4 mg/ml NBT and 0.2 ml human plasma treated with 1mM phenyl-methyl-sulphonyl-fluoride. Samples were incubated at 25^0C for 30 minutes. Allopurinol (50 µM), a xanthine oxidase inhibitor, was used in parallel samples to confirm the enzyme activity. Xanthine oxidase activity was expressed in U/L. An enzymatic activity unit was considered 1 µmole reduced NBT/minute. To calculate the enzyme activity, we used an absorption molar coefficient of 7,2 µmoles/cm^2 at 540 nm for the NBT. NBT reduction is a chemical method used to measure superoxide radical generation in various chemical (Nishikimi et al., 1972) or biological (Tarpey and Fridovich, 2001) systems.

Results obtained were utilized in standard statistical tests for calculating average values, standard deviations, Student t test and correlation coefficients (Pearson r correlation coefficient). Statistical tests were undertaken by use of a software for statistical analysis (Microsoft Excel).

RESULTS AND DISCUSSIONS

Circulating levels of most studied biochemical parameters were significantly increased in presenescent women (50-64 years old) compared with adult women (30-45 years old) (Table 1). Serum creatinine did not change, while total proteins concentrations decreased significantly in presenescent women. In senescent women (65-80 years old) serum glucose, urea, total cholesterol, triglyceride concentrations, the activities of the aminotransferases, AST and ALT, and alkaline phosphatase (ALKP), and fibrinogen level increased significantly compared with adult women, but they did not changed significantly in comparing to presenescent women. Total proteins level decreased significantly in senescent women compared with adult ones, but it is not modified compared with presenescent women. Serum creatinine and uric acid content did not change significantly in senescent women compared with those adult and presenescent. This study showed significant changes of some metabolic indicators in apparently healthy women while the hierarchy of values by age can be done inside the reference ranges considered normal for the analyzed parameters. The results suggest minor changes in the structure and functions of tissues/organs during physiologic aging.

In order to point out a possible relationship between metabolic changes and the aging process inside the reference ranges, we calculated the Pearson's r correlation coefficient using linear regression analysis. The study shoved the existence of significantly positive correlations between circulating levels of glucose, urea, uric acid, total cholesterol, triglycerides, AST, ALT, alkaline phosphatase and fibrinogen with the age of the subjects (Table 2). Under conditions of this study, for the senescent women an upper cut- off value for fibrinogen was considered at approximately 450-500 mg/dl, in order to maintain a reasonable number of subjects. On the other hand, the serum level of total proteins correlated significantly negatively with age.

Plasma xanthine oxidase activity increased significantly in presenescent and senescent women compared with adult women (Table 1), suggesting the increase of the reaction products: uric acid, $O_2^{\bullet -}$ and H_2O_2. Moreover, plasma XO significantly positively correlated with age of the subjects (Table 2), although a significant reduction in XO activity was recorded in senescent women compared with presenescent ones.

Our results are consistent with other studies (Nevaz et al., 2006; Singh et al., 2009).

Table 1 Metabolic parameters and plasma xanthine oxidase in healthy women related to age

Metabolic parameters	Age groups (years)				
	30 – 45 (Lot I)	50 – 64 (Lot II)	p	65 – 80 (Lot III)	p
Subjects number	27	25	-	20	-
Glucose (mg/dl)	85,15 ± 9,92	92,95 ± 9,19	0,0068 vs Lot I	95,26 ± 8,82	0,021 vs Lot I 0,4505 vs Lot II
Urea (mg/dl)	25,11 ± 6,23	30,26 ± 4,40	0,0017 vs Lot I	38,00 ± 6,64	< 0,0001 vs lot I 0,0001 vs Lot II
Creatinine (mg/dl)	0,85 ± 0,08	0,93 ± 0,11	0,4823 vs Lot I	0,86 ± 0,10	0,5010 vs Lot I 0,8638 vs Lot II
Uric acid (mg/dl)	3,39 ± 0,77	4,30 ± 1,13	0,0021 vs Lot I	3,85 ± 0,99	0,127 vs Lot I 0,264 vs Lot II
Total cholesterol (mg/dl)	196,66 ± 29,77	236,65 ± 32,30	<0,0001 vs Lot I	221,20 ± 46,34	0,0429 vs Lot I 0,2329 vs Lot II
Triglycerides (mg/dl)	71,35 ± 21,48	119,22 ± 43,68	<0,0001 vs Lot I	115,40 ± 35,97	<0,0001vs Lot I 0,7799 vs Lot II
AST (U/L)	16,46 ± 3,39	19,16 ± 4,76	0,0318 vs Lot I	22,15 ± 6,50	0,0008 vs lot I 0,1424 vs Lot II
ALT (U/L)	14,00 ± 3,95	22,44 ± 8,71	0,0004 vs Lot I	21,00 ± 6,84	0,0003 vs Lot I 0,5986 vs Lot II
AST/ALT	1,24 ± 0,37	0,96 ± 0,26	0,0054 vs Lot I	1,11 ± 0,22	0,2567 vs Lot I 0,0926 vs Lot II
ALKP (U/L)	51,56 ± 10,03	76,37 ± 23,36	<0,001 vs Lot I	85,80 ± 24,76	<0,001 vs Lot I 0,3201 vs Lot II
Proteins (mg/dl)	7,39 ± 0,67	7,03 ± 0,36	0,0227 vs Lot I	6,80 ± 0,44	0,0018 vs Lot I 0,9161 vs Lot II
Fibrinogen (mg/dl)	378,96 ± 52,77	443,00 ± 79,81	0,0016 vs Lot I	461,78 ± 93,39	0,0007 vs Lot I 0,528 vs Lot II
Xanthine oxidase (U/L)	0,746 ± 0,124	1,082 ± 0,264	<0,0001 vs Lot I	0,946 ± 0,104	<0,0001 vs lot I 0,0365 vs Lot II

In humans and most primates, uric acid is the final metabolite of purines catabolism. Serum urate significantly positively correlated with age (Table 2) and plasma XO (Fig. 1), suggesting the increasing of purines catabolism, via XOR, and reducing energy metabolism during physiological aging. Increased purine catabolism through XO was reported in some conditions characterized by decreased cellular energy metabolism: ischemia, hypoxia, etc. In aging there is also a diminished energy metabolism associated with an increased oxidative stress (Harman, 2001). Modulation of purine degradation rate via XDH or XO is accomplished by substrate concentration, purine metabolites. Under physiologic conditions oxidative conversion of hypoxanthine to xanthine and of xanthine to uric acid is made by XDH and, in small measure by XO. Under pathophysiological circumstances such as hypoxia, ischemia/reperfusion, XDH is oxidatively converted into XO by posttranslational reversible changes: -SH groups oxidation of XDH, and/or irreversible changes (partial proteolysis)(McCord, 1985). Xanthine oxidase intervention in purine catabolism is accompanied by supplementary generation of reactive oxygen species, $O_2^{\bullet -}$ and H_2O_2. Variations of plasma XO activity may be related to different changes that occur in aging or disease process in some tissues/organs: liver, kidney, heart, lungs, blood vessels, from which the enzyme is released through mechanisms that do not necessarily imply their lesion (Desco et al., 2002).

Table 2 Correlations between metabolic parameters and subjects age

Parameters	Linear regression (y)	R^2	r	p
Glucose (mg/dl)	0,3232x + 73,931	0,2246	0,4739	< 0,001
Urea (mg/dl)	0,3188x + 13,83	0,3983	0,6311	< 0,001
Creatinine (mg/dl)	6E-05x + 0,8466	8E-0,5	-	-
Uric acid (mg/dl)	0,0201x + 2,8401	0,0896	0,2994	< 0,05
Cholesterol (mg/dl)	0,7488x + 178,72	0,0836	0,2889	< 0,02
Trygliceride (mg/dl)	1,4261x + 26,611	0,2732	0,6226	< 0,001
AST (U/L)	0,1562x + 11,011	0,2047	0,4524	< 0,001
ALT (U/L)	0,2014x + 8,3704	0,1472	0,3836	< 0,01
ALKP (U/L)	0,9229x + 21,486	0,3473	0,5893	< 0,001
Proteins (mg/dl)	- 0,0157x + 7,9286	0,1861	- 0,4314	< 0,001
Fibrinogen (mg/dl)	2,3938x + 299,67	0,1957	0,4423	< 0,001
Xanthine oxidase (U/L)	0,0066x + 0,5731	0,1968	0,4436	< 0,001

Through correlation studies, the present work attempted to highlight the possibility that metabolic changes observed during aging could lead to increased plasma XO activity. Thus, it was shown that plasma XO activity significantly correlated with serum glucose, urea, total cholesterol and triglycerides in aging (Fig. 1). The results suggest that cellular events which led to the metabolic parameters changes are associated with impaired energy metabolism and increased purine catabolism and XO activity. It is known that aging is accompanied by increased serum glucose, insulin resistance and type 2 diabetes incidence, suggesting changes in glucose metabolism, target cells membranes, and cellular energy metabolism (Harman, 2001).

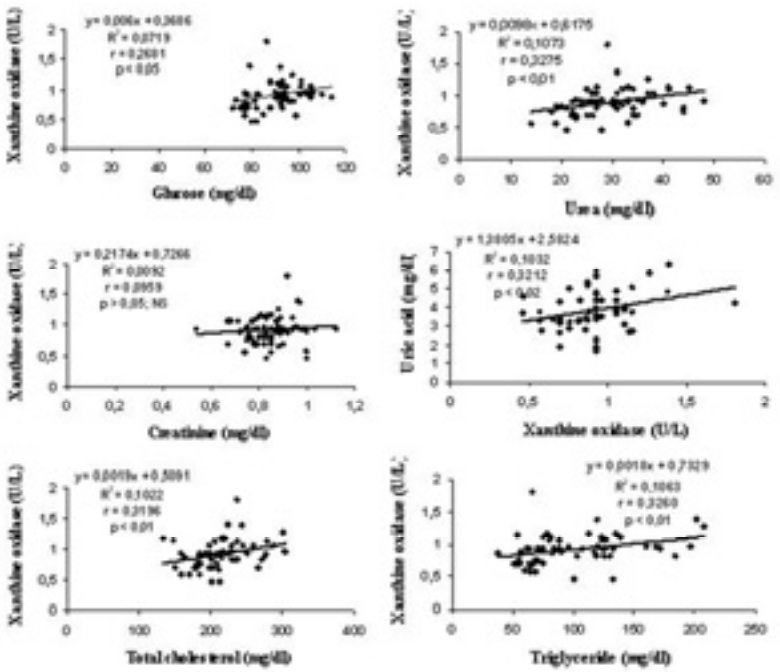

Figure 1 Correlations between plasma xanthine oxidase and metabolic parameters

Urea is the form of nitrogen excretion from proteins. In this respect, the results show a significant decrease of the serum total proteins in aging and a significant negative correlation between serum urea and total proteins ($r = - 0,2819$; $p < 0,05$). Significant positive correlation established between plasma XO and serum urea (Fig. 1) may suggest the increase of the nucleoprotein complexes degradation in aging.

Blood cholesterol comes from food processing and hepatic synthesis. In aging, a common cause of hypercholesterolemia is the increase in endogenous cholesterol. Additionally, mitochondrial oxidative degradation of fatty acids in liver is diminished in aging, influencing cellular energy status and, consequently, the rate of degradation of purines. Thus, positive correlations of the total cholesterol and triglycerides with plasma xanthine oxidase may be linked to altered lipid metabolism during aging.

Aminotransferases, AST and ALT, are enzymes that catalyze the transfer of amino groups from aspartic acid or alanine to ketoglutaric acid to produce oxaloacetic acid and pyruvic acid respectively, which are important contributors to the citric acid cycle. AST is present in the liver, cardiac muscle, skeletal muscle, kidney, brain, pancreas, lungs, leucocytes and erythrocytes with cytosolic (20%) and mitochondrial (80%) localization. It is less sensitive and specific for the liver. ALT, a cytosolic enzyme, is found in its highest concentrations in the liver, and in low concentrations in skeletal muscle and kidney. It is more specific for the liver. Both enzymes are released into blood in increasing amounts when the cell membrane is damaged (Limdi and Hyde, 2003; Gianini et al. 2005). Necrosis of the liver cells is not required for the release of the amino-transferases (Pratt and Kaplan, 2000).

Alkaline phosphatase origínate mainly in liver and bone (Limdi and Hyde, 2003). The elevations of ALKP may be physiological or pathological (biliary obstruction). Alterations in hepatic enzymes activities can point toward changes in the liver function and bile draining dysfunction. Significant increases in ALT and ALKP levels with advancing age in apparently healthy women may suggest minor alterations in the liver cells and cholestasis. Hepatocytes integrity change could be reflected in diminution of energy metabolism which leads to enhanced purine degradation by XO, and increased uric acid and oxygen reactive species ($O_2^{\bullet -}$ and H_2O_2) formation. These cellular changes could be followed by the XO release into the bloodstream. This study pointed out significantly positive correlations of ALT, AST, and ALKP with plasma XO activity (Fig. 2). No significant correlation was evidenced between plasma XO, serum total proteins and fibrinogen (Fig. 2).

An increase of xanhtine oxidase has been evidenced in vascular endothelium affected in many pathological conditions: ischemia-reperfusion, hypercholesterolemia (White et al. 1996; Daghini et al., 2006), diabetes (Desco et al., 2002), hypertension, atherosclerosis, coronary artery disease (Spiekermann et al., 2003) showing that XO is an important source of reactive oxygen species generation in age-associated diseases. Also, hyperuricaemia has been associated with hypertension (Perlstein et al 2006), increased cholesterol, diabetes, renal diseases, cardiovascular accidents (Hare and Johnson, 2003; Bergamini et al., 2009).

Senescent women showed a significant reduction of plasma XO compared with presenescent women, but significantly increased versus adult ones. Various cellular events could explain the limited increase of xanthine oxidase activity in senescent women. One of these could be the slowdown in cells metabolism in aging due to transformations undergone by cellular organelles, such as the presence of giant mitochondria with reduced number of cristae and reduced turnover (Lenaz et al., 2002), accumulation of waste products, etc.

Also, some oxidative modified biomolecules may react with XO and modulate its activity.

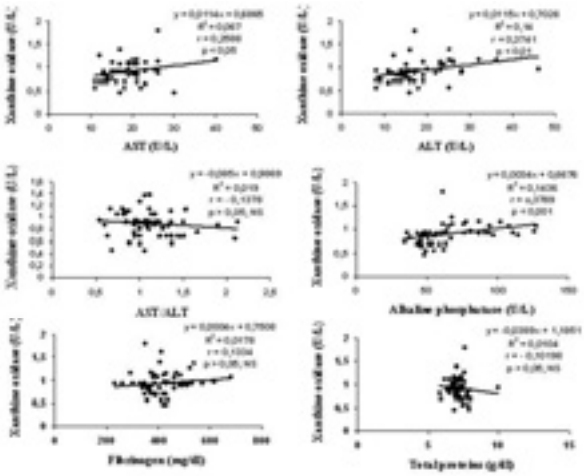

Figure 2 Correlations of plasma xanthie oxidase with serum enzymes and total proteins

Thus, xanthine oxidase may react with aldehydic products of lipid peroxidation (e.g. 4-hydroxy-2-nonenal), as well as several aldehydes derived from biogenic amines (Bounds and Winston, 1991; Xia et al., 1999). Unsaturated aldehydes, as substrates for xanthine oxidase, are inhibitors of the hypoxanthine conversion into xanthine and of the two metabolites to uric acid.

Nitric oxide and peroxynitrite ($ONOO^-$), whose production increase with aging, may represent another way to down-regulate xanthine oxidase activity (Lee et al., 2000, Godberg et al., 2000; Bagi et al, 2002). Thus, XOR can catalyse the reduction of organic as well as inorganic nitrates and nitrites to nitric oxide (NO)(Millar et al., 1998; Godberg et al., 2000; Doel et al., 2001; Harrison, 2002), acting as a source of both NO and peroxynitrite (Godberg et al., 2000; Li et al., 2001,) under hypoxic conditions in the presence of xanthine or NADH as substrates. XOR is progressively inactivated during reduction of inorganic nitrite to nitric oxide in the presence of xanthine, possible by the NO generated in the course of the reaction (Ichimori et al., 1999; Godberg et al, 2001). Inactivated enzyme may be reactivated in the presence of a sulphur generating sysytem. Apparent decrease in NO production with increasing oxygen tensions, is consistent with reaction between NO and enzyme-generated superoxyde. Thus, peroxynitrite can be produced by the action of a single enzyme, XOR, in the presence of inorganic nitrite, molecular oxygen and reducing agent, such xanthine (Godberg et al., 2000; Lee et al., 2000) Peroxynitrite can down-regulate XO activity by oxidative disruption of the molybdenum center of the catalytic site. XO inhibition by peroxynitrite may be suppressed by urate, which is a potential scavenger of $ONOO^-$ (Skinner et al, 1998). On the other side, peroxynitrite can oxidize the –SH groups of XDH converting it into XO. In conclusion, xanthine oxidase and uric acid can modulate the NO bioavailability and endothelium dependent vascular dilatation in some pathologic conditions: hypoxia, ischemia, and perhaps in aging.

Another plausible cause for the decreased plasma XO in senescent women could be the binding of circulating plasma XO to glycosaminoglycans from the external surface of endothelial cells with concomitant modification of enzyme kinetic parameters (higher K_m) and its sensibility to the allopurinol action (Adachi et al., 1993; Houston et al., 1999; Kelley et al., 2004; George et al., 2009). It was suggested that this induced form of xanthine oxidase is more important in

oxidative injury of endothelial cells than XO produced inside endothelial cells (Panus et al., 1992).

CONCLUSIONS

Our study showed significant changes of the metabolic parameters inside the normal range of values for the healthy subjects of different ages. These modifications suggest minor alteration in tissues integrity associated with lowering in the energy metabolism which could lead to enhanced purine catabolism by xanthine oxidase involvement. Plasma xanthine oxidase activity increased significantly in aging and showed a significantly positive correlation with subjects age and with metabolic parameters, such as glucose, urea, uric acid, cholesterol, transaminases and ALKP. Plasma xanthine oxidase can modulate the oxidative stress status in plasma and endothelial cells, nitric oxide bioavailability and endothelium dependent vascular dilatation.

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