CELL BIOCHEMISTRY AND FUNCTION
Cell Biochem Funct 2006; **24**: 291–298.
Published online 19 September 2005 in Wiley InterScience (www.interscience.wiley.com). **DOI**: 10.1027/cbf.1269

A central role of eNOS in the protective effect of wine against metabolic syndrome

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The positive health effects derived from moderate wine consumption are pleiotropic. They appear as improvements in cardiovascular risk factors such as plasma lipids, haemostatic mechanisms, endothelial function and antioxidant defences. The active principles would be ethanol and mainly polyphenols. Results from our and other laboratories support the unifying hypothesis that the improvements in risk factors after red wine consumption are mediated by endothelial nitric oxide synthase (eNOS). Many genes are involved, but the participation of eNOS would be a constant feature.

The metabolic syndrome is a cluster of metabolic risk factors associated with high risk of cardiovascular disease (CVD). The National Cholesterol Education Programmmes Adult Treatment Panel III (NCEPATP III) clinical definition of the metabolic syndrome requires the presence of at least three risk factors, from among abdominal obesity, high plasma triacylglycerols, low plasma HDL, high blood pressure and high fasting plasma glucose. The molecular mechanisms responsible for the metabolic syndrome are not known. Since metabolic syndrome apparently affects 10–30% of the population in the world, research on its pathogenesis and control is needed.

The recent finding that eNOS knockout mice present a cluster of cardiovascular risk factors comparable to those of the metabolic syndrome suggests that defects in eNOS function may cause human metabolic syndrome. These mice are hypertensive, insulin resistant and dyslipidemic. Further support for a pathogenic role of eNOS comes from the finding in humans that eNOS polymorphisms associate with insulin resistance and diabetes, with hypertension, with inflammatory and oxidative stress markers and with albuminuria. So, the data sustain the hypothesis that eNOS enhancement should reduce metabolic syndrome incidence and its consequences. Therefore red wine, since it enhances eNOS function, should be considered as a potential tool for the control of metabolic syndrome. This hypothesis is supported by epidemiological observations and needs experimental validation in human intervention studies. Copyright © 2005 John Wiley & Sons, Ltd.

KEY WORDS — metabolic syndrome; red wine; Mediterranean diet; endothelial nitric oxide synthase; endothelial function; insulin resistance; hypertension; obesity; HDL

ABBREVIATIONS — eNOS, endothelial nitric oxide synthase; CVD, cardiovascular disease; NO, nitric oxide; CHD, coronary heart disease; NCEPATP III, the National Cholesterol Education Programmes Adult Treatment Panel III; BH4, tetrahydrobiopterin; TAG, triacylglycerides; FFA, free fatty acids

INTRODUCTION

Ethanol and polyphenols are considered the bioactive wine components with regards to health effects. The targets, on which the study of these effects has concentrated, correspond to cardiovascular risk factors. Four main domains considered are as follow: plasma lipid metabolism, haemostatic mechanisms, endothelial function regulation, and antioxidant defence mechanisms.

Plasma lipid metabolism

Among plasma lipids, the changes in HDL appear as the most relevant after wine consumption and higher HDL levels closely correlate with decreased coronary heart disease (CHD). The increase in HDL levels has been attributed to ethanol. In a meta-analysis with

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Received 4 April 2005
Revised 2 June 2005
Accepted 28 June 2005

data from 36 different studies, Rimm *et al.*[1] clearly show a dose-dependent relationship between ethanol consumption and plasma levels of HDL, yet the mechanism of the protective effect of elevated HDL levels has not been clearly established. Reverse cholesterol transport is considered an important mechanism, but recently a new paradigm for the cardiovascular effects of HDL and estrogens that involves eNOS has been proposed.[2] These authors conclude that HDL from males, but strikingly more so in the case of HDL from pre-menopausal women because of the associated estradiol, stimulate eNOS and vasodilatation in a scavenger receptor class B type I dependent manner. The scavenger receptor acts as an HDL docking receptor, allowing for estradiol transfer. This finding is consistent with the established fact that women, before menopause, are more protected than men against CVD.[3] A transport role for HDL, mediated by scavenger receptor class B type I, has also been shown for vitamin E.[4] Assuming that the stimulation of eNOS is a key mechanism to explain the effects of increased levels of HDL in response to ethanol, it is necessary to consider that the functional expression of eNOS is strictly dependent on the presence of antioxidants that prevent NO inactivation by its reaction with superoxide anion and peroxynitrite generation. Also BH4, the eNOS co-factor, requires antioxidant defences to prevent its oxidation; its deficit leads to eNOS uncoupling with generation of superoxide anion.[5] Therefore, ethanol increases HDL levels, but necessarily requires the presence of effective antioxidant defences to enhance eNOS activity. As substantiated later, wine phenolics probably contribute to this antioxidant protective role.

Haemostatic mechanisms

In the process of haemostasis, decreased coagulation and increased fibrinolysis have been detected in relationship with wine or alcohol administration.[6,7] It is well established that alcohol consumption reduces plasma fibrinogen concentration, so diminishing the probability to form a clot.[1] Moderate alcohol increases clot lysis *in vivo* in a mouse model, by increasing the expression of tissue plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA),[8] a response also detected in human monocytes.[9] In contrast to the effect of alcohol, a deficit in eNOS function raises plasma fibrinogen.[10,11]

It has been shown that eNOS plays a key role in the process of thrombosis. In fact, red wine induces a significant reduction of stasis-induced venous thrombosis, but this effect is blunted by the eNOS inhibitor L-NAME.[12] This antithrombotic effect of wine is mainly due to polyphenols, as seen in studies that compared the effect of ethanol, ethanol-free wine and whole red wine.[13] Many studies have investigated the effect of wine or wine phenolics on platelet activation and aggregation. Several observations have demonstrated a significant inhibitory effect of red wine in platelet aggregation.[14] Since nitric oxide (NO) inhibits platelet aggregation,[15] the anti-platelet activity of wine, and of wine phenolics in particular, could be mediated by the direct enhancement of eNOS exerted by these compounds.[16–19]

Endothelial function

The endothelium plays a key role in the regulation of vascular tone and reactivity. NO is the main mediator of vascular relaxation in a process that is very sensitive to the presence of free radicals, particularly superoxide which combines with NO and generates peroxynitrite, a reaction which results in inhibition of NO-mediated processes and enhances oxidative damage by peroxynitrite. Antioxidants are required for eNOS function to protect NO, but also because of eNOS uncoupling as a consequence of reduced BH4 levels, leads to generation of superoxide.[20] Endothelin-1, a vasoconstriction mediator synthesized by the endothelium, opposes NO-induced vasodilatation. Red wine enhances the function and in some observations also the expression of eNOS, in a process dependent on phenolic antioxidants, with marked differences in activity among them.[16–20] In addition, red wine phenolics enhance endothelial function through inhibition of the secretion of endothelin-1, a vasoconstrictor agent.[21]

Together with the effects of wine phenols, it has also been reported that ethanol increases endothelial NO production in cells in culture, through modulation of eNOS expression.[22] Endothelial function can be evaluated by measuring the flow-dependent vascular reactivity, a non-invasive procedure which has shown that wine protects and enhances this eNOS-dependent regulatory mechanism.[23] The preservation of endothelial function appears in clinical observations as a requisite for cardiovascular health, particularly to prevent atherosclerosis, but the molecular mechanisms responsible for the protective effect have not been established. The present evidence suggests that the healthy effects of wine, as well as those from Mediterranean diets and polyphenols-rich foods, could be mediated, at least in part, via enhancement of eNOS function.[24,25]

Antioxidant defence mechanisms

In addition to their eNOS enhancement properties, wine phenolics are also active as antioxidants *in vitro* and *in vivo.* As mentioned before these two effects are partially related since eNOS function requires the presence of antioxidants. The effectiveness of the various phenol antioxidant species in the whole organism, their bioavailability and the antioxidant activity of their metabolites are subjects currently under investigation. The apparent target for these natural compounds is eNOS function via NO protection and gene expression. The relative contribution of gene expression and antioxidant protection to the stimulatory effect of the various wine phenolics on eNOS,[23,26] has not been established. The enhancement of endothelial function in response to acute administration of ascorbate and vitamin E,[27] but not to spirits or white wine,[26] supports the antioxidant contribution of wine phenolics. Initially, strong emphasis was given to the prevention of LDL oxidation and subsequent uptake by macrophages, as a key element in the prevention of atherosclerosis by wine antioxidant phenolics. Now, the emphasis on atherosclerosis pathogenesis has shifted to a range of factors including endothelial cell oxidative damage caused by oxidized LDL, cigarette smoke, homocysteine, lipid peroxides and inflammation.[28–30] Among the changes observed in endothelial cells after oxidative damage, the decrease in eNOS function apparently plays a central pathogenic role.

METABOLIC SYNDROME AND ITS RELATIONSHIP WITH ALCOHOL CONSUMPTION

Since the original proposition by Reaven that dyslipidemia, hypertension and hyperglycaemia commonly cluster together, a systematic analysis of these categories and others apparently related, has led to some consensus definitions of metabolic syndrome.[31] The central proposition is that several biochemical and clinical parameters recognized as CVD risk factors do cluster together. The definition of metabolic syndrome by the NCEPATP III report considers the clustering of at least three of five cardiovascular risk factors, for all of which definite abnormal values are proposed. These five principal risk factors are as follows: waist circumference, plasma triacylglycerols, plasma HDL cholesterol, blood pressure and fasting blood glucose. Similar risk factors have been selected by other organizations such as the World Health Organization (WHO) and the American Association of Clinical Endocrinologists to define the metabolic syndrome.[32] In order to explore the underlying pathophysiological causes for this multivariate condition, several phenotypic characteristics of the metabolic syndrome were evaluated by applying multivariate factor analysis; the results showed 3 and 4 factor domains, classified as obesity, blood pressure, lipids and central obesity.[33] These authors could account for approximately 60% of the variance in 11-original variables. Their study involved approximately 3000 subjects included in the Hypertension Genetic Epidemiology Network; metabolic syndrome was present in 34% of black and 39% of white participants. Obesity, with its relationship to lipids and insulin, was found to be the dominant factor in metabolic syndrome.

Research has shown a favourable insulin profile in light-to-moderate alcohol consumers, when compared with non-drinkers and heavy drinkers. The increased insulin sensitivity associated with light-to-moderate consumption appears to be the consequence of the body mass index (BMI) and the central adiposity profile in studies of CV risk factors.[34] For Reaven, the key to overcoming metabolic syndrome is an increase in insulin sensitivity.[35] In a study in which alcohol intake and insulin sensitivity, measured by the clamp technique, were correlated, the conclusion was reached that alcohol consumption was independently and positively associated with insulin-mediated glucose uptake.[36] And in a cross-sectional study in severely obese subjects, it was shown that light-to-moderate alcohol consumption was associated with lower prevalence of type 2 diabetes, together with reduced insulin resistance and a more favourable risk factor profile. The conclusion stated in this work was that light-to-moderate alcohol consumption should not be discouraged in the severely obese. Also in this study, wine drinkers had lower fasting TAGs, lower insulin and higher HDL cholesterol when compared with spirit drinkers.[37] In a prospective study of 49 324 women, it was shown that light-to-moderate drinking was not associated with weight gain over an 8-year period. However, the result was not so clear for African–American women.[38] In another prospective study done in Denmark with a sample of 2916 men and 3970 women, it was found that after 10 years, the moderate consumption of alcohol, beer and spirits was associated with later high waist circumference, whereas moderate-to-high consumption of wine apparently had the opposite effect.[39] The apparent protective effect of wine on abdominal obesity may be related to our recent finding that wine phenolics interact with Glut-4 glucose transporters in adipocytes and thereby inhibit glucose transport.[40] From studies addressing the relationship among alcohol consumption

and the prevalence of metabolic syndrome directly, in Sweden and USA, it can be concluded that moderate alcohol consumption is significantly and inversely associated with metabolic syndrome, as shown for several components of the metabolic syndrome such as low serum HDL cholesterol, elevated serum TAGs, high waist circumference, hyperinsulinemia and hypertension. Sex differences are observed and wine drinkers exhibit a more favourable pattern of metabolic and clinical parameters, as well as a better lifestyle, than beer and spirit drinkers.[41–43]

PATHOGENESIS OF THE METABOLIC SYNDROME, A CENTRAL ROLE FOR eNOS

In their report on metabolic syndrome Grundy *et al*. discuss the present views on its pathogenesis.[32] Obesity, insulin resistance, dyslipidemia and hypertension are all conditions for which pathogenic mechanisms have been extensively explored, and the results from such studies are likely to be relevant when considered in the context of the metabolic syndrome. Endocrine factors, a pro-inflammatory state and the role of the renin-angiotensin system, have also been discussed in relationship with pathogenesis. Oxidative stress has also been considered.[44] Yet, the overall picture does not lead easily into a unifying hypothesis or interpretation, capable of simultaneously explaining the various components that cluster in this highly prevalent syndrome.

That single gene defects may lead to pleiotropic phenotype modifications is a common biological observation. Thus, it is possible that the metabolic syndrome is a consequence of single gene modifications. There is an evidence in support of a central role for defects in NO production in the pathogenesis of many of the cardiovascular risk factors that cluster in the metabolic syndrome. Strikingly, Cook *et al*. made a very interesting observation in eNOS null mice.[11] These animals present several phenotype changes that mimic the cluster of cardiovascular risk factors that define the human metabolic syndrome including: hypertension, insulin resistance, hypertriacylglyceridemia, elevated fibrinogen and several other changes. The same authors also showed that a high fat diet triggers a marked increase in blood pressure and insulin resistance in eNOS ($+/-$) mice.[45] Consistent with the findings reported by Cook *et al*. are the reports in humans that eNOS polymorphisms are associated with several signs characteristic of metabolic syndrome including insulin resistance and type 2 diabetes,[46] hypertension,[47] inflammatory and oxidative stress markers,[48] together with albuminuria, that is another abnormality often found in metabolic syndrome.[49] Also in support of the role played by eNOS in the pathogenesis of metabolic syndrome, it has been found that erectile dysfunction,[50] known to arise from decreased NO concentration,[51] is associated with the metabolic syndrome.

There are findings that describe other genetic defects associated with metabolic syndrome, but they do not provide, for the moment, arguments as strong as those shown for eNOS. For example, a mutation in a mitochondrial tRNA gene leads to a cluster of metabolic defects partly similar to metabolic syndrome.[52] Also the peroxisome proliferator-activated receptor (PPAR) has been involved in metabolic syndrome pathogenesis.[53] And signs that partly resemble metabolic syndrome have recently been observed in subjects with mutations in PPARγ.[54] Insulin-sensitizing thiazolidinedione related drugs are PPARγ agonists and are used to decrease insulin resistance. Interestingly, it was demonstrated that PPARγ ligands stimulate NO release from the endothelial cell.[55,56]

Regular physical activity is associated with favourable modification of metabolic syndrome parameters. The mechanisms mediating the protective effects of exercise are not clearly defined, but it has been shown that exercise training, in many animal and human studies, augments endothelial function in both large and small blood vessels.[57] Recent human studies also indicate that exercise training may improve endothelial function by up-regulating eNOS protein expression and phosphorylation.[58–60] In another finding suggestive of a central role of eNOS in metabolic syndrome, eNOS null mice were shown to have reduced mitochondrial oxidative capacity in slow-twitch skeletal muscle, and also reduced spontaneous physical activity.[61]

POTENTIAL ROLE OF RED WINE, AS eNOS ENHANCER, IN THE CONTROL OF THE METABOLIC SYNDROME

We have reviewed the arguments that support a central role for eNOS in the positive health effects of moderate wine consumption, an effect largely attributed to wine polyphenols. Similarly, we review the evidence linking eNOS dysfunction with the cluster of cardiovascular risk factors recognized as metabolic syndrome. The obvious question stemming from the experimental data is to what extent moderate red wine consumption contributes to the control of metabolic syndrome manifestations and its long-term health consequences. In this context we propose a potential role for red wine, as eNOS enhancer, in the control

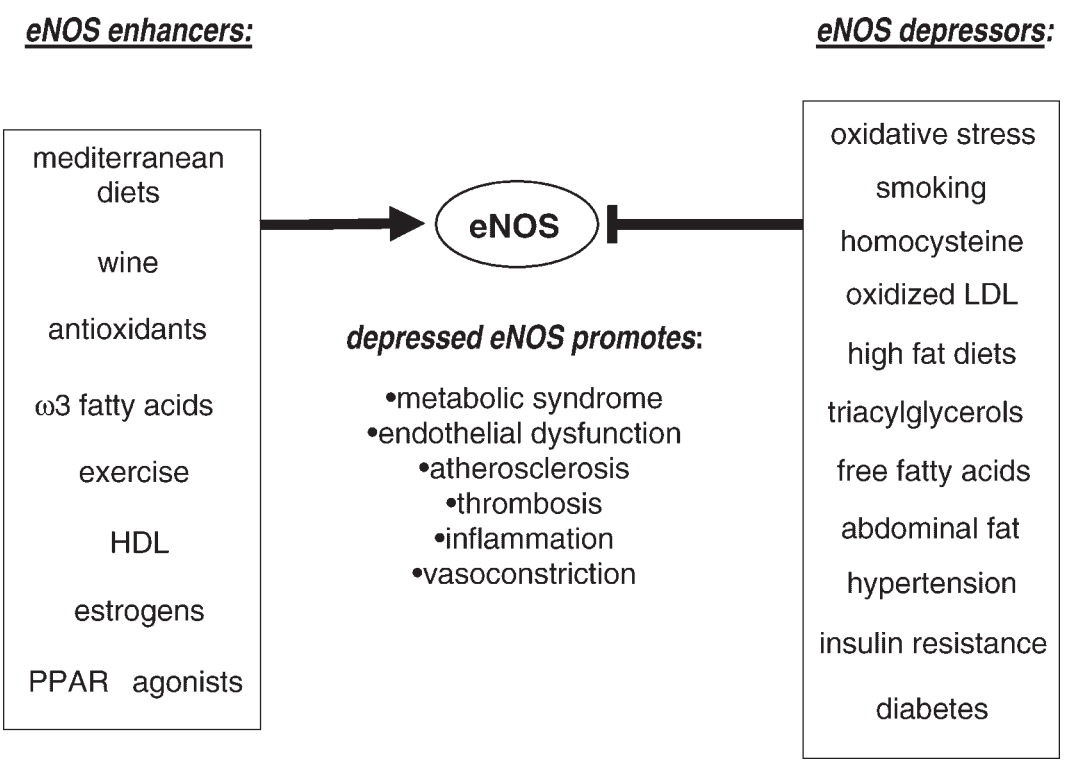

Figure 1. Factors which are capable of enhancing or depressing eNOS function via gene expression, enzyme regulation, substrate availability, product stability or others. Decreased eNOS would result in metabolic syndrome, as well as in other biological changes, some of which are indicated

of the metabolic syndrome. Indirect evidence outlined here, from epidemiological studies that correlate wine and alcohol consumption with metabolic syndrome, support our proposition (Figure 1).

On the whole, the evidence linking eNOS with the metabolic syndrome and with the individual risk factors that characterize it, strongly suggests that the search for a unifying theory to account for the metabolic syndrome should focus systematically on eNOS and its functional role. In fact, Parks and Booyse have emphasized the potential role of eNOS as the 'cardioprotective protein' mediating alcohol and polyphenols cardioprotective activities.[30] Similarly, the relationship among eNOS function, type 2 diabetes and vascular disease is actively being explored.[62]

In the context of atherogenesis, the anti-inflammatory effect of red wine consumption has been characterized; TNF-alpha-induced adhesion of monocytes to endothelial cells was virtually abolished after red wine consumption.[63] Also red wine decreases interleukin-1alpha (IL-1alpha), C-reactive protein (hs–CRP), as well as monocyte and endothelial adhesion molecules.[64] Since NO reduces leukocyte adhesion to vascular endothelium, wine phenolics enhancement of eNOS activity could be involved in these effects. Other interesting relationships among eNOS, metabolic syndrome, endothelial cell inflammation and PPARγ agonists, have recently been described.[65]

Additional support for the hypothesis that red wine could contribute to the control of metabolic syndrome comes from the evidence that metabolic syndrome is associated with oxidative stress. Reduction of oxidative stress may lead not only to decreased oxidative damage to biological structures but also to changes in signalling pathways responsive to oxidative stress that might be involved in the pathogenesis of the metabolic syndrome.[44,66] eNOS relates to oxidative stress because it can generate superoxide when uncoupled, and because NO requires antioxidant protection. Thus, malfunction of eNOS might result from oxidative stress affecting NO, or from uncoupled eNOS, generating more superoxide and less NO.

Wine phenolics enhance eNOS function measured as NO production, they increase eNOS gene expression and also lead to enzyme activation.[16,19,67–69] We and others have provided evidence that a

Mediterranean like diet and also red wine consumption, i.e. high phenolic intake conditions, improve endothelial function in human subjects.[23] Wine is a very efficient vehicle to provide antioxidant phenols in human subjects, an effect associated with adherence to Mediterranean diets.[24] A recent trial of a Mediterranean-style diet in patients with the metabolic syndrome has shown improvements in endothelial function and a decrease in vascular inflammation markers, providing further support for our proposal.[70] So, polyphenols present in red wine and in fruits and vegetables, abundant in Mediterranean diet, as well as exercise and PPARγ agonists, increase NO release in endothelial cells, improve endothelial function and decrease metabolic syndrome risk factors. Metabolic syndrome unquestionably constitutes a serious challenge for human health today, but perhaps effective therapeutic and preventive measures are already available. The establishment of a pathogenic theory, which we believe needs to be explored in connection with the hypothesis that eNOS function is deficient in metabolic syndrome, would help in unifying criteria for the prevention of the health consequences of this pleiotropic cluster of homeostatic disorders.

ACKNOWLEDGEMENTS

This work was supported by projects FONDEF D03I-1047 and PBMEC-UC 2004–2005.

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