Red wine polyphenols affect the collagen composition in the aorta after oxidative damage induced by chronic administration of CCl₄

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Short title

Polyphenols influence collagen composition in vessel wall

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Summary
Increased amount of collagen type I. and decreased amount of type III. is described in various pathological processes in the vascular wall. Polyphenols were shown to have protective effect on endothelium, decrease blood pressure and prevent oxidative damage induced by various stimuli. Tetrachlormethane (CCl₄) is a toxic substance with known negative systemic effects induced by free radicals. Chronic administration of CCl₄ for 12 weeks led to an increase of collagen type I. and a decrease of type III. in the wall of aorta. Parallel administration of red wine polyphenols significantly reduced the increase of collagen type I., at the same time the content of type III. rose to the level above controls. After 4 week of spontaneous recovery no changes were observed. If polyphenols were administered during the recovery period, there was a decrease of type I. and an increase of type III. collagen content in the aorta. It can be concluded that polyphenols have the tendency to lower the amount of type I. and to increase the proportion of type III. collagen in the wall of the aorta. These changes are significant in prevention or in regression of changes induced by chronic oxidative stress. This effect of polyphenols is most likely the result of their influence on MMP-1 and TIMP activities through which they positively influence the collagen types I. and III. content ratio in the vascular wall in favor of the type III. collagen.

Key words: Red wine, Polyphenols, Collagen, Aorta, CCl₄

Introduction

Polyphenolic compounds represent a wide spectrum of substances in foods including lignins (walnuts, cereals), proantocyanins (vine, pine bark), antocyanins (fruits, vegetables), isoflavons (soya beans), catechins (thee, vine), tannins (tea, nuts),
quercetin (grapes, vine, onion) and naringenin (citrus fruits) (Mandelová 2005).

Epidemiologic studies indicate that high intake of polyphenols in vegetables and fruits is connected with decreased cardiovascular diseases. Mechanisms that would explain the mentioned observation remain unclear. It is supposed that flavonoids improve functions of endothelial cells and inhibit platelets aggregation (Vita 2005). One of the important facts is the decreased oxidation of LDL in the presence of flavonoids (Miyagi et al. 1997, Hayek et al. 1997, O’Byrne et al. 2002).

Short lasting administration of polyphenols from red wine leads to a decrease of blood pressure in normotensive rats. This hemodynamic effect is connected with the increase of endothelium-related relaxation and induction of genes expression of inducible NO-synthase and COX-2 in the vascular wall (Diebolt et al. 2001). Bernátová et al. (2002) reported by polyphenols evoked faster and deeper decrease of blood pressure in experimental hypertension induced by chronic inhibition of NO synthesis. They reported also decreased hypertrophy of vascular walls, improved endothelium-related relaxation responses and reduction of the increased vasoconstrictor reactivity. Preventive effects of red wine polyphenols on increased blood pressure, myocardial fibrosis, reduction of vascular wall remodeling and improved vascular functions were also demonstrated in experimental hypertension model (Pecháňová et al. 2004). The protection of functional and structural changes was ascribed to the increased NO production. However, the significance of modulation of oxidative stress by polyphenols was also pointed out.

Application of polyphenols in prevention and therapy of neoplastic processes is also investigated. Nakazato et al. (2005) described rapid apoptosis of myeloid leukemia cells activated by catechin through modulation of reactive oxygen species production.
Collagens are a heterogeneous group of structurally related proteins of the extracellular matrix. There are roughly 27 types of collagens divided according to the structure and size of their α chain and tissue distribution (Boot-Handford et al. 2003). The aortic wall contains filaments of collagen, smooth muscle cells and fibers of elastin as basic structural components. It is known that the collagen fibers bear the circular tension and elastin exerts both longitudinal and transversal support. Stiffness of the vascular wall is connected with the loss of elastic tissue and the increase of collagen content (Silver et al. 2001). Main collagens present in the aortic wall are of type I. and III. (Satta et al. 1995).

Increased number of cells producing type I. collagen has been described in every type of atherosclerotic lesion in man (Andreeva et al. 1997). It had been shown that collagen type I. supports calcification of vessels in vitro (Watson et al. 1998). It also may play a role in neoangiogenesis in the plaque (Jackson and Jenkins 1991) and in organization of thrombi (Rekhter et al. 1996). Moreover, there are proofs about a relationship between collagen type I. accumulation and the seriousness of coronary artery restenosis after angioplasty (Pickering et al. 1996). However, insufficient formation of type III. collagen is linked to the occurrence of aneurysms in the abdominal aorta and cerebral arteries without (Majamaa et al. 1992, Anderson et al. 1996) or with connection to atherosclerosis (Kuga et al. 1998).

Tetrachlormethan (CCl₄) is a toxic substance from which a trichlormethyl radical is formed by P-450. Further process of detoxication includes trichlormethylperoxyl radical formation that produces lipoperoxidation and oxidative stress (International Programme on Chemical Safety, 1999). CCl₄ is frequently used for experimental liver cirrhosis development (Zwart et al. 1998). Recently, it has been applied as a model of oxidative damage to vascular endothelium (Babál et al. 2006)
The aim of the presented work is to evaluate how the polyphenols influence the content of collagen type I. and III. in the wall of aorta in experimental animals exposed to chronic oxidative stress produced by administration of CCl₄.

**Materials and Methods**

*Animals*

All procedures and experimental protocols were approved by the *Ethical Committee of the Institute of Normal and Pathological Physiology SAS*, and conform to the *European Convention on Animal Protection and Guidelines on Research Animal Use*. Male Wistar rats, 3 months old, were divided into six groups, 8 animals in each. The preventive experiment lasting for 12 weeks consisted of four groups: the control group, the group receiving CCl₄ 0.5 ml/kg of body weight twice a week subcutaneously in a 1:1 solution with olive oil, the group receiving dried red wine extract Provinols™ (40 mg/kg/day) in drinking water and the group receiving Provinols™ + CCl₄. In the recovery experiment, the initial 12 weeks of CCl₄ treatment were followed by 4 weeks of spontaneous recovery in the first, and recovery with Provinols™ administration in the second group of animals. To make sure that each animal received the complete dose of Provinols™, calculated amount of Provinols™ was given to each rat in the appropriate volume of water. Daily water consumption was estimated individually for every animal one week before the experiment. During the experiment, water consumption was controlled and Provinols™ concentration in the drinking fluid was adjusted, if necessary. All animals were housed at a temperature of 22-24 °C and fed with a regular pellet diet *ad libitum*. 
The red wine extract dry powder Provinols™ was kindly provided by Mr. D. Ageron (Société Francaise de Distillerie, Vallont Pont d’Arc, France). Provinols™ polyphenols content has already been reported (Diebolt et al. 2001) and it was (in mg/g of dry powder): proanthocyanidins 480, total anthocyanins 61, free anthocyanins 19, catechin 38, hydroxycinnamic acid 18, flavonols 14.

**Histology**

The thoracic aorta, carotid, pulmonary and renal arteries were fixed 24 hours in 10 % formalin, routinely processed in paraffin and 5 μm thick slices were cut perpendicularly to the vessel axis and stained with hematoxylin and eosin. The slides were evaluated in a Leica light microscope (Leica Systeme, Wetzlar, Germany).

**Collagen type I. and III. evaluation**

Deparaffinized and rehydrated 5 μm thick slices were stained with a modified technique with picrosirius red as follows: the slides were submerged in 0.2 % phosphomolybden acid for clearing the cytoplasm, then the slides were stained with 0.1 % sirius red F3BA in a saturated water solution of picric acid for 90 min. The slides were washed 2 min in 0.01 N HCl, dehydrated and mounted. The findings were documented with a digital photographic camera GC-X3E (JVC, Japan) and evaluated with ImageJ software (National Institute of Health, Bethesda, USA). Threshold values were determined for the particular colors of spectrum: from 0 to 35 for the red color corresponding to the type I. collagen, from 45 to 110 for the green color corresponding to the collagen type III. (Fig. 1). The numbers of pixels of each color were counted and the percentage of the whole crosssectional area was calculated.
Statistics

The results were expressed as mean±standard error, statistically analyzed by one-way ANOVA with Keuls-Neumann test.

Results

The group of animals administered CCl₄ had increased content of collagen type I. in the wall of aorta (56,3 ± 2,2 %). Parallel administration of polyphenols in the group CP lead to decreased amount of type I. collagen in the aorta (27,2 ± 3,4 %) when compared to control. The group P administered polyphenols contained higher amount of collagen type I. (38,3 ± 3,8 %) then CP and lower then the control group K (43,6 ± 3,7 %), but the differences were not statistically significant (Fig. 2).

The group C had decreased content of collagen type III. (20,7 ± 1,6 %). Parallel administration of polyphenols with CCl₄ resulted in its higher content in aortic wall (52,7 ± 3,9 %). The group P contained 39,4±3,9 % and lower content was found in the control group K (29,1 ± 2,6 %) (Fig. 3).

In the animals after spontaneous recovery following the intoxication with CCl₄, the content of collagen type I. in the wall of aorta was the highest (56,5 ± 2,3 %). If polyphenols were administered during the recovery phase, the amount of collagen type I. (8,2 ± 1,0 %) was the lowest (Fig. 4). Collagen type III. content was the lowest (18,6 ± 1,8 %) in the CR group. Conversely, administration of polyphenols during the recovery period resulted in its highest content (71,2 ± 3,2 %) (Fig. 5).

Discussion
The study shows that administration of CCl₄ increases the amount of collagen type I. and on the contrary, decreases the content of collagen type III. in the wall of the aorta. At present, tetrachlormethane is most frequently used for liver cirrhosis induction through the mechanism of oxidative stress (Zwart et al. 1998). It had been confirmed that chronic (10 weeks) administration of CCl₄ increased the amount of collagens type I. and III. together with fibronectin in the liver. The oxidative damage by tetrachlormethane does not concern only the liver. Described are e.g. toxic damage of bone marrow and the spleen or the kidneys (Singh et. al. 1990). A direct relation between liver cirrhosis induced by CCl₄ and vascular changes has been reported (Castro et al. 1993, Zhang et al. 1997). Recently, toxic effect on vascular endothelium has been reported (Babál et al. 2006).

We have found that subcutaneous administration of tetrachlormethane lead to a decreased content of collagen type I. and an increase of collagen type III. in the of aorta. Increased amount of type I. collagen in blood vessels is considered an unfavorable factor. An increase in its production is observed in various pathological processes in blood vessels, like atherosclerosis (Andreeva et al. 1997) or coronary stenosis (Lafont et al. 1999). In contrast, the decreased amount of collagen type III. is attributed to reduced elasticity of the vessels (Silver et al. 2001) and aneurysms formation (Kontusaari et al. 1990, Majamaa et al. 1992, Anderson et al. 1996).

Sirius red F3BA dissolved in the saturated picric acid solution stains collagens and viewed under polarized or fluorescent light the color of collagen fibers depends on their thickness (Allon et al. 2006). Detailed study of combined usage of picrosirius red with hue analysis documented suitability of this method for collagens evaluation (Rich and Whittaker 2005). The reliability of such analysis is supported by the results
obtained by immunohistochemistry and expression of collagen type I. and type III. mRNA (Pauschinger et al. 1999).

Components of extracellular matrix are in a dynamic balance in the organism (Bissel 2001). Remodeling of the extracellular matrix includes both, the degradation and removal of its components, as well as the production and deposition of the newly synthesized components. Homeostasis of these processes influences the preservation or the changes of structure or function of the tissue (Liu and Connolly 1998). Matrix metalloproteinases (MMP) mediate the resorption of extracellular material, while the creation of extracellular matrix depends mainly on the production of collagens (Mauch 1998). Under normal physiological conditions the activity of metalloproteinases is regulated by tissue inhibitors of proteinases (Nagase and Woessner, 1999). Loss of the control of MMP activity for whatever reason may result in various diseases like arthritis, atherosclerosis, aneurysm, nephritis and fibrosis (Woessner 1998).

MMP-1 is a collagenase that splits collagens type I., II. and III., while type III. collagen is split more effectively than the other types of collagen (Ohuchi et al. 1996). This enzyme is not produced by healthy endothelium. However, its presence is confirmed in atherosclerotic plaques (Nikkari et al. 1995) and increased amounts of MMP-1 are found in aneurysms (Lesauskaite et al. 2001). On the contrary, decreased amount of MMP-1 prevents development of vascular lesions (Wilson et al. 2003). Tissue inhibitors of proteinases (TIMP) play an important role in maintenance of the dynamic balance of collagen matter. Their increased presence acts as a protective factor from aneurysm rupture (Allaire et al. 1998) and reduces atherosclerotic changes (Rouis et al. 1999).
Polyphenols were shown to increase TIMP expression (Lambert and Yang 2003). According to our results, the effect of polyphenols on collagen types content ratio in the wall of the aorta is more expressed in the toxic damage induced by tetrachloromethane then in the normal control tissue. Evaluation of the control groups (with or without polyphenols) showed only a moderate shift of the ratio in favor of the type III. collagen, when compared to the toxic groups. This difference might result from the damaged control mechanisms in CCl₄ intoxication. Under physiological conditions, the regulatory molecules like TIMP, are able to maintain the balance between the particular units of the extracellular matrix, which could explanation the less effective performance of polyphenols in the control groups. Chronic toxicity of CCl₄ results in serious systemic damage that has a significant effect on the dynamic equilibrium between components of the extracellular matrix. As had been mentioned above, polyphenols inhibit the synthesis of MMP-1 (Oak et al. 2004) and increase the formation of TIMP (Lambert and Yang 2003). Through the preference of collagen type III. as the substrate for MMP-1 (Ohuchi et al. 1996), the polyphenols influence the collagen types I./III. ratio in favor of collagen type III. By this activity the polyphenols enhance their protective effect on blood vessels from oxidative damage caused by tetrachlormethane.

**Conclusion**

Subcutaneous application of CCl₄ increases the amount of collagen type I. and on the contrary decreases the amount of type III. in the wall of the aorta. Red wine polyphenols lead to reduced content of collagen type I. and increase the proportion of collagen type III. in the aortic wall. This effect is enhanced after previous oxidative
damage when compared with the control, and also after 4 weeks of recovery. The
effect of polyphenols is most likely the result of their influence on MMP-1 and TIMP
activities through which they positively influence the collagen types I. and III. content
ratio in favor of the type III. collagen.

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**Figure legends:**

Figure 1. Aorta stained with picrosirius red showing digitalization of the findings.  
**a.** Aorta from CCl₄-treated animal, with the perivascular fat tissue (asterix).  
**b.** The wall of aorta deprived of perivascular fat and intima. Both types of collagen (type I. is originally red and type III. is green) are captured.  
**c.** Digitally subtracted red color detecting collagen type I.  
**d.** Digitally selected green color detecting collagen type III.  
Picrosirius red, fluorescence light, original magnification 200x.

Figure 2. Collagen type I content in the aorta after chronic intoxication with CCl₄ (C) and the preventive effect of parallel administration of polyphenols (CP). Control group (K), polyphenols alone (P). ** p<0.01 compared to K.

Figure 3. Collagen type III content in the aorta after chronic intoxication with CCl₄ (C) and the preventive effect of parallel administration of polyphenols (CP). Control group (K), polyphenols alone (P). * p<0.05, ** p<0.01, *** p<0.001 compared to K.

Figure 4. Collagen type I content in the aorta after chronic intoxication with CCl₄ followed by 4-week reparation phase without (CR) and with administration of polyphenols (CRP). Control group (K), polyphenols administration alone (P). ** p<0.01 compared to K, **** p<0.0001 compared to K.
Figure 5. Collagen type III content in the aorta after chronic intoxication with CCl₄ followed by 4-week reparation period without (CR) and with polyphenols administration (CRP). Control group (K), polyphenols administration alone (P). * p<0.05, *** p<0.001, **** p<0.0001 compared to K.
Fig. 3

Collagen type III.
preventive experiment

Fig. 4

Collagen type I.
recovery experiment

Fig. 5

Collagen type III.
recovery experiment