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Study of potential systemic oxidative stress animal models for the evaluation of antioxidant activity: status of lipid peroxidation and fat-soluble antioxidants

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Abstract

Although many compounds have already been tested in-vitro to determine their antioxidant profile, it is necessary to investigate the in-vivo effect of potential antioxidants. However, representative models of systemic oxidative stress have been poorly studied. Here, different potential systemic oxidative stress animal models have been investigated. These included a vitamin E-deficient rat, a diabetic rat and an atherosclerotic rabbit model. Plasma/serum malondialdehyde was measured as a parameter of oxidative damage. Plasma/serum fat-soluble antioxidants were determined as markers of antioxidant defence. We demonstrated that vitamin E-deficient rats were not suitable as a model of systemic oxidative stress, whereas diabetic and atherosclerotic animals showed increased systemic oxidative damage, as reflected by significantly augmented plasma/serum malondialdehyde. Moreover, plasma coenzyme Q_9 increased by 80% in diabetic rats, confirming systemic oxidative stress. In view of these observations and economically favouring factors, the diabetic rat appeared to be the most appropriate systemic oxidative stress model. These findings have provided important information concerning systemic oxidative stress animal models for the in-vivo study of antioxidants.

Introduction

Oxidative stress has been associated with ageing and many chronic diseases such as atherosclerosis, diabetes and cancer. Antioxidants could have a protective role in delaying or preventing oxidative stress, and so much scientific research has focused on the antioxidative effects of foods, extracts and pure compounds. Many compounds have been tested already in-vitro to determine their antioxidant profile (Cos et al 2002, 2003). As these in-vitro tests do not account for problems of malabsorption, distribution, metabolism and excretion, it is indispensable to investigate the activity of 'promising' antioxidants in-vivo. However, studying oxidative stress and antioxidant potency in-vivo involves some difficulties. Apart from the development of a battery of well-validated assays to assess different oxidative stress parameters, it is necessary to have a representative oxidative stress animal model. Although induction of oxidative stress in animals has been frequently reported in the literature, further investigation of potential oxidative stress animal models is necessary. Most publications about oxidative stress induction report only local effects at specific organs (e.g. the liver) (Ikeda et al 1998; Ramirez-Tortosa et al 2001; Aoki et al 2002; Fischer et al 2002). Therefore, the establishment of an animal model of systemic oxidative stress, implicating significant changes of oxidative stress parameters in body fluids, such as plasma, instead of only effects observed in certain tissues, is an important issue.

Several strategies have been described in the literature to induce oxidative stress in laboratory animals. Induction of oxidative stress may be achieved by exposure to a toxic agent such as carbon tetrachloride (CCl_4) or paraquat (Nomura & Yamaoka 1999; Teselkin et al 2000; Aoki et al 2002). A second strategy is dietary-induced oxidative stress, which may be caused by nutritional imbalance due to either an antioxidant deficient diet or a pro-oxidant overload diet. Examples are a vitamin E-deficient diet (Chow 1991; Ramirez-Tortosa et al 2001; Yamagishi et al 2001) and an iron overload diet, respectively (Fischer et al 2002). Since oxidative stress is

associated with many diseases, it can also be studied by using a pathological animal model such as a diabetic rat model (Young et al 1995; Maritim et al 2003) or an atherosclerotic rabbit model (Aikawa et al 2002; Griendling & FitzGerald 2003). Models in which radical scavenging mechanisms are directly impaired constitute a fourth strategy to induce oxidative stress in-vivo. This may be achieved through inhibition of glutathione synthesis by buthionine sulphoximine (BSO) (Ichiseki et al 2006). Finally, other approaches to induce oxidative stress in experimental animals have been reported including exercise- or radiation-stressed animal models and transgenic animal models (Sentürk et al 2001; Melov 2002; Abou-Seif et al 2003).

In this study, different potential systemic oxidative stress animal models were compared: CCl_4-treated rats as a model of toxicant-induced oxidative stress, vitamin E-deficient rats as a model of dietary-induced oxidative stress, and diabetic rats and atherosclerotic rabbits as models of oxidative stress-related pathologies. Due to several compromising factors and negative preliminary observations in plasma with respect to oxidative stress, the CCl_4 oxidative stress model was not studied further. In contrast, the vitamin E-deficient, diabetic and atherosclerotic animal models were investigated to select the most appropriate systemic oxidative stress animal model. Plasma or serum malondialdehyde (MDA), which is an important parameter of oxidative lipid damage, and fat-soluble antioxidant levels (retinol, α -and γ-tocopherol and coenzyme Q) were measured by using our previously optimized and validated assays (Hermans et al 2005). In this way, the suitability of the above-mentioned models for the study of systemic oxidative stress was assessed.

Materials and Methods

Animals

For the CCl_4-treated rat model, male Wistar rats (200–250 g) were procured from Charles River (Brussels, Belgium). Rats were administered an intra-peritoneal injection of a mixture of CCl_4 and olive oil (1:1, 4 mL kg^{-1} body weight) once a day, for two days (Nomura & Yamaoka 1999; Teselkin et al 2000; Pande & Shukla 2001). An equivalent amount of olive oil was given to the control animals. Twenty-four hours after the second injection, the rats were killed and blood samples were collected.

For the vitamin E (vit E) deficient animal model, three-week-old male Sprague-Dawley rats were obtained from Charles River (Brussels, Belgium). The rats were randomly divided into two groups. One group had free access to a vit E-deficient (< 0.5 mg kg^{-1}) diet (Ssniff, Germany) for 20 weeks, whilst the second group had free access to a normal semi-synthetic (200 mg kg^{-1} vit E) diet (Ssniff, Germany) for 20 weeks. After this time the rats were killed and blood samples were collected.

With respect to the diabetic rat model, male Wistar rats (200–225 g) were procured from Charles River (Brussels, Belgium). Type I diabetes was induced by a single intra-peritoneal injection of streptozotocin (Sigma-Aldrich, USA), 60 mg kg^{-1}, diluted in a 20 mM citrate buffer, pH 4.5. The presence of diabetes was confirmed after one week by measuring blood glucose levels using a portable blood sugar monitor (FreeStyle, Disetronic Medical Systems, The Netherlands). Rats with a glycaemia less than 15 mmol L^{-1} were excluded. A group of non-diabetic control animals, which received a single intra-peritoneal injection of pure citrate buffer, 20 mM, pH 4.5, was also included. Seven weeks after diabetes induction, the rats were killed and blood samples were collected.

For the atherosclerotic model, 12-week-old male New Zealand white rabbits obtained from the Rijksstation voor Kleinveeteelt (Merelbeke, Belgium) were fed a 0.3% cholesterol diet (Altromin, Germany) for 20 weeks. A group of rabbits receiving normal (cholesterol-free) diet (Altromin, Germany) for 20 weeks was also included. Besides the cholesterol content, the composition of both diets was identical. In week 20 blood samples were collected.

The Ethical Committee of the University of Antwerp approved all experiments.

Sample preparation

After anaesthetizing the rats with sodium pentobarbital (Nembutal, 60 mg kg^{-1}, intra-peritoneally) blood samples were collected from the carotic artery in 1-mL tubes (Eppendorf, Germany) containing potassium EDTA (30 μL 7.5% EDTA/mL blood sample), which led to animal death. After centrifugation (1000 *g*, 13 min, 4°C) plasma samples were stored at −70°C.

The rabbit blood samples were collected from the central ear artery in Vacutainer SST blood collection tubes for serum analysis (Becton Dickinson, Belgium). After 30 min, serum was centrifuged (1300 *g*, 10 min).

Haemolysed samples were discarded.

Lipid peroxidation assay

The in-vivo lipid peroxidation status was determined by a recently optimized and fully validated method (Hermans et al 2005). Briefly, this method quantifies MDA after reaction with thiobarbituric (TBA) in acid and heat conditions, and the resulting pink fluorescent complex is analysed by HPLC-fluorescence detection.

Fat-soluble antioxidant status

For the determination of retinol, α- and γ-tocopherol and coenzyme Q plasma levels, a recently optimized and validated HPLC-coulometric detection method was used as described by Hermans et al (2005).

Statistical analysis

Statistical differences were established using the unpaired two-tailed Student's *t*-test. A value of $P < 0.05$ was considered significant. The results are expressed as means ± s.e.m. The number of animals is represented by the letter n.

Results

CCl_4-treated rat model

Preliminary experiments were conducted on four control and four CCl_4-treated rats. Two CCl_4-stressed animals died. The MDA levels of the remaining animals were 0.45 ± 0.02 μM for controls (n=4) and 0.51 ± 0.02 μM (n=2) for the CCl_4-treated rats.

Oxidative stress status in vitamin E-deficient rat model

After receiving a vit E-deficient diet for 20-weeks, no significant change ($P > 0.05$) was observed in the lipid peroxidation status of rat plasma. Plasma MDA concentrations averaged 0.43 ± 0.02 μM for vit E-deficient rats (n = 11) and 0.41 ± 0.04 μM for control animals (n = 8) (Table 1).

As expected, the concentration of the fat-soluble antioxidant α-tocopherol was significantly reduced in rats fed the vit E-deficient diet ($P < 0.001$, n = 7). In addition, γ-tocopherol levels were significantly reduced in vit E-deficient animals (control: 65 ± 6 ng mL^{-1} vs vit E deficient: < 4.5 ng mL^{-1}(limit of detection), $P < 0.001$, n = 7). Furthermore, plasma retinol levels were significantly lowered ($P < 0.05$, n = 7). The endogenous fat-soluble antioxidant coenzyme Q_9, which is the dominant coenzyme Q species in rats, was also measured. Although the plasma concentration of coenzyme Q_9 in vit E-deficient rats showed a 20% increase as compared with the control group, this difference was statistically not significant ($P > 0.05$, n = 7) (Table 1).

Oxidative stress status in diabetic rat model

The plasma levels of MDA were significantly increased in diabetic rats (Table 2). In control animals (n = 6), the plasma MDA concentration amounted to 0.54 ± 0.03 μM, whereas in the diabetic group (n = 8) the mean plasma MDA was 0.80 ± 0.02 μM.

With respect to the concentrations of fat-soluble antioxidants, there was no significant difference ($P > 0.05$) of α-tocopherol and γ-tocopherol levels in plasma of control and diabetic rats, while retinol levels were significantly reduced in diabetic animals ($P < 0.05$). In contrast, the mean concentration of the endogenous antioxidant coenzyme Q_9 was increased by 80% in the diabetic animal group ($P < 0.05$) (Table 2).

Table 1 Plasma MDA and fat-soluble antioxidant concentrations in vitamin E-deficient rat model

	Control	Vitamin E deficient
MDA (μM)	0.41 ± 0.04 (n = 8)	0.43 ± 0.02 (n = 11)
Fat-soluble antioxidants:		
α-Tocopherol (μg mL^{-1})	13.73 ± 1.02 (n = 7)	0.25 ± 0.03*** (n = 7)
γ-Tocopherol (ng mL^{-1})	65 ± 6 (n = 7)	< 4.5*** (n = 7)
Retinol (ng mL^{-1})	748 ± 54 (n = 7)	544 ± 53* (n = 7)
Coenzyme Q_9 (ng mL^{-1})	192 ± 23 (n = 7)	238 ± 25 (n = 7)

Values represent means ± s.e.m. *$P < 0.05$, ***$P < 0.001$.

Table 2 Plasma MDA and fat-soluble antioxidant concentrations in diabetic rat model

	Control (n = 6)	Diabetic (n = 8)
MDA (μM)	0.54 ± 0.03	0.80 ± 0.02***
Fat-soluble antioxidants:		
α-Tocopherol (µg mL^{-1})	7.57 ± 0.56	8.64 ± 0.69
γ-Tocopherol (ng mL^{-1})	210 ± 17	158 ± 22
Retinol (ng mL^{-1})	595 ± 40	498 ± 20*
Coenzyme Q_9 (ng mL^{-1})	186 ± 28	337 ± 45*

Values represent means ± s.e.m. *$P < 0.05$, ***$P < 0.001$.

Table 3 Serum MDA and fat-soluble antioxidant concentrations in atherosclerotic rabbit model

	Control	Atherosclerotic
MDA (μM)	0.99 ± 0.09 (n = 6)	2.28 ± 0.26*** (n = 11)
Fat-soluble antioxidants:		
α-Tocopherol (μg mL^{-1})	2.99 ± 0.95 (n = 5)	9.29 ± 3.28 (n = 10)
γ-Tocopherol (ng mL^{-1})	72 ± 20 (n = 5)	521 ± 122** (n = 10)
Retinol (μg mL^{-1})	1.30 ± 0.06 (n = 5)	1.49 ± 0.15 (n = 10)

Values represent means ± s.e.m. **$P < 0.01$, ***$P < 0.001$.

Oxidative stress status in atherosclerotic rabbit model

In atherosclerotic rabbits a significant increase ($P < 0.001$) of lipid peroxidation was observed as compared with control rabbits. Serum MDA amounted to 2.28 ± 0.26 μM (n = 11) in hypercholesterolaemic animals and 0.99 ± 0.09 μM (n = 6) in control animals (Table 3).

Although the mean α-tocopherol concentrations in the atherosclerotic rabbits were approximately three times higher than in the control group, this numerical difference was statistically not significant due to the large variability in α-tocopherol levels in atherosclerotic animals. However, a significant increase in γ-tocopherol was observed in atherosclerotic rabbits ($P < 0.01$). The levels of retinol in serum of control and atherosclerotic groups were statistically not different (Table 3). Coenzyme Q_9 was not detectable in the rabbit serum.

Discussion

In this study, different animal models were investigated to establish a systemic oxidative stress animal model. The CCl_4-induced oxidative stress model has been used for decades. In many reports, the local effects of potential antioxidants on the liver of CCl_4-stressed animals have been evaluated (Kolhir et al 1996; Teselkin et al 2000; Pande & Shukla 2001; Gupta et al 2004). Although in some studies significant changes of oxidative stress parameters in body fluids have been observed (Teselkin et al 2000; Kadiiska et al 2005), our preliminary experiments with this hepatotoxic chemical did not show systemic oxidative stress. Several compromising factors, such as CCl_4-induced liver toxicity, which is a potential health risk for the laboratory personnel, an increased drop-out of animals and the absence of any effect on plasma lipid peroxidation status (MDA), resulted in an early termination of these experiments. In contrast, a vitamin E-deficient rat model, a diabetic rat model and an atherosclerotic rabbit model were intensively studied and compared to select the most appropriate systemic oxidative stress model. The fat-soluble antioxidant

status and the levels of MDA were determined in plasma or serum samples by using our previously optimized techniques (Hermans et al 2005). In addition to MDA, F2-isoprostanes are important markers of oxidative lipid damage. However, inclusion in a screening battery is hampered by complex sample preparation and sophisticated quantification techniques, involving GC-MS or LC-MS/MS. For the determination of MDA an HPLC system with fluorescence detection was used, which permits a sensitive and specific quantification of the MDA–TBA adduct. In contrast, the interference of other TBA-reactive substances by simple spectrophotometrical measurement of the MDA complex is an important problem (Chirico 1994), although this technique is still often used for MDA determination (Duarte et al 2001; Ermis et al 2004; Tütüncüler et al 2005). Fat-soluble antioxidants were determined by HPLC combined with multi-channel coulometric detection, which permits analysis of different compounds in one run providing not only the retention time but also the electrochemical characteristics as unambiguous identification.

The vitamin E-deficient rat model was not suitable for induction of systemic oxidative stress because changes in plasma MDA were not observed after 20 weeks of feeding a vitamin E-deficient diet. Although α- and γ-tocopherol levels significantly decreased, this change merely reflected dietary deficiency. The plasma concentration of the endogenous antioxidant coenzyme Q_9, the main coenzyme Q form in rats, remained unchanged while plasma retinol decreased by 30%. Diminished liver stores and plasma levels of retinol due to vitamin E deficiency have been reported before (Napoli et al 1984). Moreover, a 30–40% loss of retinal vitamin A after prolonged (30 weeks) vitamin E deficiency has been observed, leading to impaired visual function (Goss-Sampson et al 1998). These reduced retinol levels may be due to an increased oxidative destruction of vitamin A in the absence of vitamin E (Napoli et al 1984; Basu & Dickerson 1996; Thompson & Manore 2005). The lack of a significant change in rat plasma lipid peroxidation status after prolonged nutritional deficiency of vitamin E, probably the most important inhibitor of lipid peroxidation in membranes and lipoproteins, was an unexpected result. However, it is conceivable that the presence of other antioxidants in-vivo may provide a possible explanation for this observation. Indeed, apart from the vitamin E regeneration system, which involves different antioxidants including ascorbic acid, glutathione, lipoic acid and ubiquinol, the selenoenzyme glutathione peroxidase complements the antioxidant function of vitamin E. It reduces the lipid hydroperoxides formed, thus diminishing the requirement for vitamin E. This glutathione peroxidase system responds compensatively to oxidative stress, which may be responsible for the lack of significant augmentation of lipid peroxidation in vitamin E deficient animals (Chow 1991, 2004).

On the other hand, plasma or serum MDA levels were significantly increased in the diabetic rat and the atherosclerotic rabbit model, indicating increased systemic oxidative lipid damage. These animal models were studied as potential systemic oxidative stress models because diabetes and atherosclerosis are both associated with increased production of reactive oxygen species and oxidative stress plays a major role in the pathophysiology of these diseases (Brownlee 2001; Guzik et al 2002; Yorek 2003; Harrison et al 2003). In the atherosclerotic rabbits, serum MDA and γ-tocopherol levels were significantly increased. Although the mean α-tocopherol concentrations were approximately three-times higher than in the control group, this difference was statistically not significant because of a large variability in α-tocopherol levels in the atherosclerotic animals. The increased serum vitamin E concentration was probably due to the cholesterol-enriched diet. Since vitamin E is transported by lipoproteins, the changes in serum vitamin E could be due to an altered transport capacity (Vandewoude et al 1987; Simon et al 1997). Coenzyme Q_9 was not detectable in the rabbit serum. Previous studies have shown that coenzyme Q_{10} was the dominant coenzyme Q homologue in rabbit tissues (Matsura et al 1991; Albano et al 2002). Nevertheless, in most rabbits coenzyme Q_{10} serum levels were below the limit of detection, thus making any comparison impossible.

In the diabetic rat model, a significant increase was observed in plasma MDA, but also in the endogenous antioxidant coenzyme Q_9. This was an important observation since augmented coenzyme Q levels have been reported as an adaptive response in conditions of increased oxidative stress (Ernster & Dallner 1995; Turunen et al 2004). Plasma levels of retinol were significantly reduced, while vitamin E levels were unchanged under diabetic conditions, which was in agreement with previously reported results (Young et al 1995; Basu & Basualdo 1997). Besides reduced plasma retinol levels, increased hepatic stores of vitamin A have been reported in diabetic animals, which have been attributed to a disturbance in the secretion of retinol binding protein from the liver to the circulation (Basu & Basualdo 1997). However, the underlying cause for reduced circulatory status of vitamin A in diabetic animals is not understood clearly.

In view of economical aspects, including time and costs for induction of oxidative stress, the diabetic rat model appeared the most interesting. Systemic oxidative stress was observed within seven weeks after diabetes induction. In contrast, a period of 20 weeks was used for the atherosclerotic rabbits and the vitamin E-deficient rats. For the latter systemic oxidative lipid damage was not observed even after this long induction period. Regarding the atherosclerotic rabbit model it should be mentioned that higher dietary cholesterol levels (up to 2%) may shorten induction of systemic oxidative stress (Aikawa et al 2002; Oubina et al 2002). Nevertheless, with respect to overall costs, rats are preferred to rabbits.

Conclusions

In this study different animal models were investigated as potential models of systemic oxidative stress, which implicates significant changes of oxidative stress parameters in body fluids, such as plasma or serum. The results were limited to the evaluation of the oxidative lipid damage and the status of fat-soluble antioxidants. These results have given important information on the use of animal models as models of systemic oxidative stress for the evaluation of antioxidants in-vivo. However, it should be emphasized that the measurement of additional parameters of oxidative damage, such as oxidative DNA damage, might be interesting to characterize fully the effect of the antioxidant tested.

We have demonstrated that CCl_4-treated or vitamin E-deficient rats were not suitable as animal models of systemic oxidative stress, whereas the diabetic and atherosclerotic animals showed increased systemic oxidative damage, as reflected by augmented levels of plasma and serum MDA. In particular, apart from increased plasma MDA, the endogenous antioxidant coenzyme Q_9 increased by 80% in plasma of diabetic animals, reflecting an oxidative stress status. Following these observations and the economically favouring factors, the diabetic rat was the most appropriate model for systemic oxidative stress. These findings have provided important information concerning systemic oxidative stress animal models for the in-vivo study of antioxidants.

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