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Effect of Quercetin on Daunorubicin-Induced Heart Mitochondria Changes in Rats

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Summary

Cancer therapy with daunorubicin is limited by its cardiotoxicity. It has been suggested that daunorubicin-induced free radical generation can be involved. The precise molecular mechanism of daunorubicin-induced cardiotoxicity is still not well understood but it is believed that mitochondria play an important role in this process. It has been reported that flavonoids with antioxidant properties may prevent anthracycline-induced cardiotoxicity. In this work, we investigated the effects of daunorubicin and quercetin on mitochondrial enzyme activities such as ATPase, glutathione peroxidase (GPx) and glutathione reductase (GR). Moreover, we also studied the changes of outer mitochondrial membrane using synchronous fluorescence spectra. The actitivity of ATPase and GR were significantly increased after daunorubicin application. Pretreatment with quercetin significantly alleviated this increase. On the other hand, GPx activity was significantly decreased and quercetin prevented this decrease. Treatment with quercetin alone had no significant effect on the enzyme activity studied. Quercetin also completely prevented daunorubicin-induced changes in fluorescence of the outer mitochondrial membrane. In conclusion, our data indicate that quercetin may be useful in mitigating daunorubicin-induced cardiotoxicity.

Key words
Mitochondria • Antioxidant enzymes • Daunorubicin • Quercetin • Synchronous fluorescence spectra

Introduction

Daunorubicin, an anthracycline antibiotic, is one of the most frequently used antineoplastic agents for the treatment of leukemias and solid tumors. However, clinical use of anthracyclines is limited by various unwanted effects, of which cardiotoxicity is the most serious (Singal and Kirshenbaum 1990, Allen 1992).

In spite of extensive investigation, the mechanism of the cardiotoxicity of anthracycline antitumor drugs has not yet been completely elucidated. Several hypotheses have been proposed to explain anthracycline-induced cardiotoxicity, including a calcium overload (Chacon *et al.* 1992), free radical production (Malisza and Hasinoff 1996, Kotamraju *et al.* 2000) or "non-free radical" theory (Jeyaseelan *et al.* 1997, Arai *et al.* 1998).

Much existing information on the cardiotoxicity of anthracyclines points to the mitochondrial membrane as the main target of cellular toxicity (Praet and Ruysschaert 1993, Link *et al.* 1996). It was found that the toxicological activation of daunorubicin requires addition

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of NADH in the case of cardiac mitochondria (Nohl 1988). On the other hand, NADH was ineffective when liver mitochondria were exposed to adriamycin. Furthermore, it was reported that exogenous mitochondrial NADH dehydrogenase (Sokolove 1994) is associated with the cytosolic face of the inner membrane in the heart, but not in liver mitochondria (Nohl 1987). It was suggested that mitochondria catalyze a single electron reduction of anthracyclines in the presence of extramitochondrial NADH (Lin *et al.* 1991). Later, the key role of the exogenous NADH dehydrogenase associated with complex I of heart mitochondria was documented in the process of one electron reduction of anthracyclines, giving rise to oxygen radical formation *via* autoxidation of adriamycin semiquinones (Nohl *et al.* 1998).

Flavonoids are a group of about 4000 naturally occurring compounds that are ubiquitously present in foods of plant origin. They have a broad scale of biological effects and it is believed that many of them are due to their antioxidant properties. Flavonoids may exert antioxidant effects as free radical scavengers, hydrogen-donating compounds, singlet oxygen quenchers or metal ion chelators (Rice-Evans *et al.* 1995, Korkina and Afanas´ev 1997). One of the most abundant natural flavonoids, present in a large number of fruits and vegetables, is quercetin (Kuo *et al.* 1998).

Treatment with anthracyclines can by associated with different types of cardiotoxicity including acute toxicity, subacute toxicity, chronic toxicity and delayed toxicity. Our aim was to investigate the ability of quercetin to affect daunorubicin-induced acute cardiotoxicity in rats. Therefore, we studied the effect of daunorubicin and quercetin, both alone and in combination, on glutathione peroxidase and glutathione reductase activity. Furthermore, changes in synchronous fluorescence spectra of outer mitochondrial membrane were also studied.

Methods

Animals and drug administration

Male Wistar rats, weighing 190-200 g were used. They had free access to a commercial balanced stock diet and water. The animals were randomly divided into 4 groups (n=10) which ran simultaneously: 1) rats treated intragastrically with saline in a dose 5 ml/kg (C - control group); 2) animals treated intraperitoneally with daunorubicin (Daunoblastina, Pharmacia & Upjohn) in a dose 15 mg/kg (D); 3) animals treated intraperitoneally with daunorubicin in a dose 15 mg/kg; 60 min before daunorubicin application, quercetin (Sigma, Germany) in a dose 100 mg/kg was given intragastrically (D+Q); 4) only quercetin (100 mg/kg) was given intragastrically as an internal control (Q).

The dose of quercetin (100 mg/kg) was chosen on the basis of our preliminary experiments in which quercetin prevented daunorubicin-induced elevation of plasma concentrations of lactate dehydrogenase and creatine kinase (unpublished results).

Mitochondrial isolation and enzyme activities

Twenty-four hours after daunorubicin application, the tested animals were decapitated and myocardial mitochondria were isolated according to the method of Mela and Seitz (1979). The medium for mitochondria isolation consisted (in mol/l) of mannitol 0.225, sucrose 0.075, and EDTA 1.0. The glutathione reductase activity (GR) was determined according to Calberg and Mannerick (1985) glutathione peroxidase (GPx) according to Flohe and Gunzler (1984). The content of proteins in isolated mitochondria was estimated by the method of Hartree (1972).

Measurement of synchronous fluorescence spectra

Sample preparation: Mitochondria after isolation were resuspended in medium contained (in mmol/l) mannitol 0.225, sucrose 0.075, K_2HPO_4 10.0, TRIS-HCl 10.0 and EDTA 0.2 (pH 7.4) at an equal protein concentration 28 mg/l and then diluted to a final protein concentration 56 μg/l.

Synchronous fluorescence spectra (SFS) were measured by means of an improved fluorescence spectrometer Perkin-Elmer Model 3000 equipped with PC. The wavelength scan speed of both monochromators was 240 nm/min. The constant wavelength difference was kept at $\Delta\lambda$=70 nm. The measured data were processed automatically by computer. All spectral measurements were carried out using 1 cm quarz cuvette (Dubayová *et al.* 1997).

Statistical analysis

All results are presented as the mean ± S.D. Statistical significances were determined with Student's t-test. Values of $P<0.05$ were considered to be significant.

Results

Enzyme activity

In the D group, GPx activity was decreased significantly to 61.8 % of the control ($p<0.001$). In the Q group the value at 24 h after treatment was 93.1 % of the

control values. Although the combination of both drugs (D+Q group) partially prevented the decrease in enzyme activity, values reached only 76.5 % of the control group. Despite that, differences in enzyme activity between D and D+Q groups were also significant ($p<0.01$) (Fig. 1).

On the other hand, in the D group, the GR activity significantly increased to 159.8 % of the control value ($p<0.001$). In the D+Q group, the GR activity was also significantly increased to 138.5 % in comparison with the control group ($p<0.01$), but the differences in GR activity between D and D+Q groups were also significant ($p<0.01$). Quercetin alone had no significant effect on GR activity (Fig. 2).

Synchronous fluorescence spectra (SFS)

SFS is a graphical record of fluorescence intensity versus excitation and emission wavelength Synchronous flurescence spectrum ($\Delta\lambda$=70 nm) of native heart mitochondria is characterized by a marked peak at λ=348 nm (proteins) and by a smaller flat maximum at λ=385 nm. This spectrum served as a standard for comparison with spectra of mitochondria from animals treated with daunorubicin and/or quercetin.

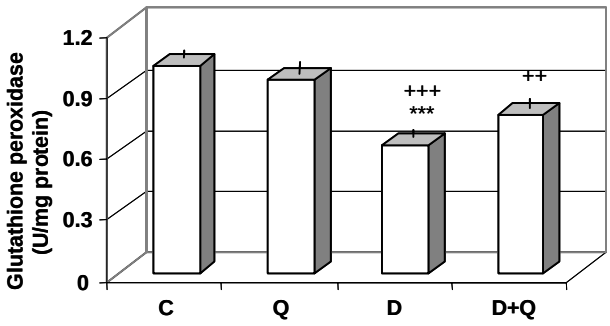

Fig. 1. *Effect of quercetin (Q), daunorubicin (D) and their combination (D+Q) on mitochondrial glutathione peroxidase activity (mean ± S.D., n = 10) in comparison with control animals (C).* $^{+++}p<0.001$ *vs C,* $^{++}p<0.01$ *vs C,* $^{***}p<0.001$ *vs D+Q*

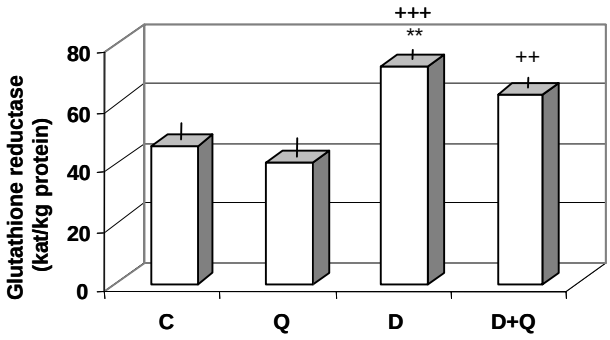

Fig. 2. *Effect of quercetin (Q), daunorubicin (D) and their combination (D+Q) on mitochondrial glutathione reductase activity (mean ± S.D., n = 10) in comparison with control animals (C).* $^{+++}p<0.001$ *vs C,* $^{++}p<0.01$ *vs C,* $^{**}p< 0.01$ *vs D+Q*

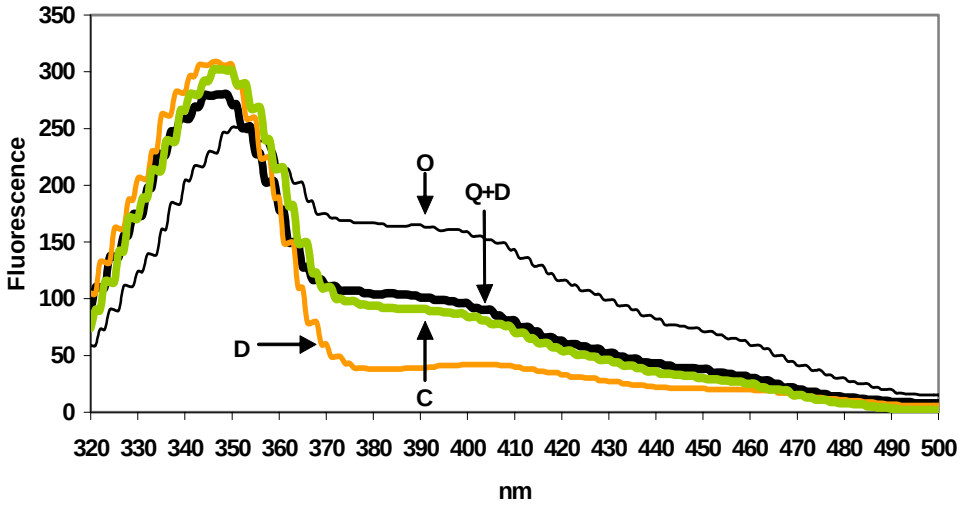

Fig. 3. *Synchronous fluorescence spectra $\Delta\lambda$=70 nm of rat heart mitochondria isolated from daunorubicin-treated animals (D), quercetin-treated animals (Q) and animals treated with combination of daunorubicin and quercetin (D+Q). C - control animals.*

SFS of mitochondria isolated from animals treated *in vivo* by daunorubicin in comparison with standard SFS show a different shape. The second peak is shifted from λ=385 nm (control) to λ=405 nm, with lowered fluorescence intensity. This is supposed to be a consequence of the daunorubicin effect on the outer mitochondrial membrane.

Quercetin also strictly changed the shape of SFS of isolated mitochondria. The principal maximum at λ=348 nm is shifted to λ=355 nm and its intensity is strongly reduced. On the other hand, the second peak reached a maximum at λ=399 nm and fluorescence intensity was greatly increased in comparison with the control group

Synchronous fluorescent spectrum of isolated rat heart mitochondria from animals treated with combination of D+Q is very similar to the control mitochondria (Fig. 3).

Discussion

Anthracycline antibiotics, despite their potential cardiotoxicity are often used in the treatment of a wide range of human malignancies. There are certain modalities, however, anthracycline-induced cardiotoxicity (AIC) may be influenced by a reduction of the cumulative dose, a method of medical chronobiology, the use of new, low-toxicity anthracycline derivates, or liposome-encapsulated drugs. Although several hypotheses explaining the mechanisms of anthracycline-induced cardiotoxicity have been put forward, most of them suggested a crucial role of free radicals and iron ions (Tarasiuk *et al*. 1998, Minotti *et al*. 1999). In biological systems, daunorubicin is known to produce highly reactive free radicals (Doroshow 1983, Mansat-de Mas *et al*. 1999) and antioxidant enzymes play a critical role in inactivation of these radicals (Kaul *et al.* 1993). It is well known that cardiomyocytes are particularly susceptible to free radicals because the activity of antioxidant defense mechanisms is lower than in other tissues (Doroshow *et al.* 1980, Singal and Kirshenbaum 1990). According to the "free radical" theory, several compounds with antioxidant or iron-chelating properties have been shown to reduce free radical formation (van Vleet *et al.* 1980, Herman *et al*. 1994) but only ICRF-187 (dexrazoxane), an EDTA analogue was used in clinical practice (Weiss *et al.* 1999).

Several years ago, it was reported that flavonoids, mainly monohydroxyethylrutoside, provide excellent protection against AIC *in vitro* and *in vivo* with no effect on antitumor activity of doxorubicin (Hüsken *et al*. 1995, van Acker *et al.* 1997). Furthermore, no toxic effects of flavonoids were observed in contrast to dexrazoxane which was toxic to the bone marrow (Koning *et al*. 1991). Moreover, it was reported that the application of monohydroxyethylrutoside once a week provides sufficient protection against AIC (van Acker *et al*. 2000). As mentioned above, quercetin is one of the most abundant natural flavonoids. Its antioxidant effect was documented in many *in vitro* and *in vivo* experimental studies (Chen *et al.* 1990, Mojžiš *et al.* 2001, Miroššay *et al.* 2001). Furthermore, quercetin is known as an excellent metal chelator (Afanas´ev *et al.* 1989). Recently, it was cofirmed that both antiradical and chelating effects are involved in the protective effect of quercetin (Sestili *et al*. 1998, Cheng and Breen 2000).

In biological systems, anthracyclines are known to produce free radicals (Doroshow 1983, Horenstein *et al.* 2000), and antioxidant enzymes play a critical role in the detoxication of these radicals. It has been suggested that GPx may play an important role in protecting the heart from peroxidative attack (Doroshow *et al.* 1980). We found that daunorubicin significantly decreased the activity of GPx. Our results are in agreement with those of Sazuka *et al*. (1989) who found a similar decrease in antioxidant enzyme activity in mice after anthracycline treatment. Recently, this effect of anthracyclines was also confirmed by Li and Singal (2000) who reported a significant decrease of GPx activity two hours after anthracycline administration and this decrease continued up to 24 hours. Quercetin treatment partially prevented this daunorubicin-induced decrease in GPx activity. In the D+Q group enzyme activity was found to be considerably higher than in the D group. Furthermore, quercetin treatment has also been reported to significantly improve the decrease of GPx activity induced by oxidative stress (Erden *et al.* 2001, Nagata *et al*. 1999).

Effect of anthracycline antibiotics on glutathione -dependent antioxidant enzymes including GR has been studied in many *in vitro* and *in vivo* experiments. However, the results of these studies are controversial. It was documented that chronic administration of adriamycin was associated with inhibition of GR activity (Hino *et al*. 1985, Gustafson *et al.* 1993). On the other hand, Thayer (1988) found no changes in GR activity in rats chronically treated with adriamycin. Moreover, Robinson *et al.* (1989) even observed an increase in GR activity in animals subjected to multiple doxorubicin treatment. Furthermore, effect of daunorubicin on GR activity has not been previously described. In our

experiments, GR activity was significantly increased in daunorubicin-treated animals. We suggest that increased GR activity may be a consequence of daunorubicin-induced oxidative stress. This hypothesis is supported by the finding that oxidative stress elevates the activity of glutathione reductase (Koul *et al*. 2001, Leutner *et al.* 2001, Senf *et al*. 2002). In animals treated with a daunorubicin+quercetin combination, there was a significant decrease in GR activity as compared to the daunorubicin-treated animals. We suggest that this can be a consequence of a quercetin antioxidative effect.

Biomolecules including amino acids, structural proteins, enzymes and coenzymes, vitamins, lipids and porhyrins present in various cells exhibit endogenous fluorescence. Autofluorescence of proteins in biological membranes is caused by amino acids with an aromatic side chain – tryptophan (λ_{ex}=280 nm; λ_{em}=350), tyrosine (λ_{ex}=275 nm; λ_{em}=300 nm) and phenylalanine (λ_{ex}=260 nm; λ_{em}=280 nm). The endogenous fluorophore that is speculated to play a role in transformation that occurs in carcinogenesis and oxidative processes, is coenzyme NADH (λ_{ex}=290 nm, 350 nm; λ_{em}=440, 460 nm) (Ramanujam 2000).

From the fluorescence point of view, the mitochondrial membrane is a mixture of endogenous fluorophores. To resolve many fluorescence components without previous physical separation, a synchronous fluorescence spectroscopy was applied. The synchronous fluorescence spectrum is more broken than the emission spectrum and it provides more information. The synchronous spectrum is considered to be the characteristic „fingerprint“, because it is unique for a given mixture and is characterized graphically as one unit.

Synchronous fluorescent spectrum ($\Delta\lambda$=70 nm) of mitochondria represents a simplified graphical definition of the outer mitochondrial membrane**.** It characterizes the structural arrangement of individual membrane fluorophores – mainly the protein fluorescence (λ=348 nm) and fluorescence of NADH (λ=385 nm). Each chemical or physical change on the surface of the mitochondria is associated with changes in this spectrum (Kušnír *et al*. 2000).

The quinone structure of anthracycline permits these compounds to act as an electron acceptor, the transfer being mediated by various flavoprotein enzymes. Mitochondrial enzymes (e.g. NADH dehydrogenase) have been shown to activate daunorubicin to form the semiquinone radical and superoxide anion. Furthermore, during reverse electron transport daunorubicin inhibited the reduction of NAD^+ to NADH. In our experiments, we found a decrease in the spectral peak at λ=385 nm in daunorubicin-treated animals. Because the spectral peak at 385 nm is typical for the reduced form of NADH, we conclude that it is closely related with changes of NAD^+/NADH ratio which is a consequence of daunorubicin metabolism (Davies and Doroshow 1986). On the other hand, quercetin, as an antioxidant molecule can favor the reduced form of NADH and enhance its peak. Combination of both drugs probably eliminates the effects of each other and fluorescence in the D+Q group is similar to that in control animals.

In summary, daunorubicin caused significant changes in the activities of antioxidant enzymes and synchronous spectra. Quercetin reverted the effects of daunorubicin probably due to its antioxidant effects. We suggest that this compound offers a perspective for lowering the cardiotoxicity of daunorubicin.

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