British Journal of Pharmacology (1996) 119, 671-676

Protective action of hydroxyethyl rutosides on singlet oxygen challenged cardiomyocytes

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1 The effect of a standardized mixture of β-hydroxyethyl rutosides against oxidative damage in singlet oxygen-challenged isolated cardiac myocytes from adult rats was investigated. The morphology of the myocytes was evaluated as an indicator for cell viability (elongated, rod shaped cells vs. hypercontracted, rounded cells). The determination of the production of thiobarbituric acid reactive substances served as an indicator for lipid peroxidation.

2 Exposure to singlet oxygen which was generated by photo-excitation of rose bengal (10^{-7} M) reduced the number of rod shaped (vital) cardiomyocytes by 78.5±2.5% and increased the production of thiobarbituric acid reactive substances by 1180±150% in comparison to incubation with control buffer.

3 Coincubation of the cells with β-hydroxyethyl rutosides (concentration range: 6.7 pg ml^{-1} to 670 μg ml^{-1}) increased the number of rod shaped cardiomyocytes after exposure to singlet oxygen in a dose-dependent bell-shaped manner. A significant protective effect was observed at β-hydroxyethyl rutosides concentrations ranging from 0.67 ng ml^{-1} to 67 ng ml^{-1}.

4 In spite of their protective action, β-hydroxyethyl rutosides did not reduce the accumulation of thiobarbituric acid reactive substances, used as an indicator for lipid peroxidation.

5 The data suggest that β-hydroxyethyl rutosides exert a protective action against oxygen radical-induced damage of cardiac myocytes at very low concentrations without interfering with lipid peroxidation.

Keywords: Cardiac myocytes; reactive oxygen species; rutosides; thiobarbituric acid reactive substances

Introduction

Membrane phospholipid peroxidation induced by free oxygen radicals is regarded as a major mechanism of injury in a variety of diseases (Marx, 1987). Among these, free oxygen radicals are considered to play an important role in the pathogenesis of myocardial ischaemia-reperfusion injury (Guarnieri *et al.*, 1980; Gauduel & Duvelleroy, 1984). The involvement of free oxygen radicals in this pathological condition indirectly has been supported by protective effects of antioxidants such as superoxide dismutase, catalase and other free radical scavengers in ischaemia-reperfusion models (Gauduel & Duvelleroy, 1984; Gross *et al.*, 1986; Näslund *et al.*, 1986; Ver Donck *et al.*, 1988; Werns *et al.*, 1986; Woodward & Zakaria, 1985).

Flavonoids are naturally occurring polyphenolic substances with free radical scavenging activity (Sorata *et al.*, 1984; Husein *et al.*, 1987; Robak & Gryglewski, 1988). Recent data showed an inverse correlation between dietary flavonoid intake and mortality from coronary heart disease (Hertog *et al.*, 1993). This phenomenon has been linked up with the ability of flavonoids to inhibit oxidative modification of low density lipoproteins (De Whalley *et al.*, 1990; Mangiapane *et al.*, 1992; Frankel *et al.*, 1993) and to exert an antithrombotic action (Gryglewski *et al.*, 1987). β-Hydroxyethyl rutosides (oxerutins, Venoruton), in the following abbreviated to HR, is a standardized mixture of hydroxyethyl derivatives of rutin (3-O-[6-O-alpha-L-rhamnosyl-beta-D-glucosyl]quercitin). The drug is widely used for the relief of oedema and related symptoms in patients with chronic venous insufficiency (Pulvertaft, 1983; De Jongste *et al.*, 1989). Its therapeutic effect has been primarily attributed to a reduction of microvascular permeability (Gerdin & Svensjoe, 1983; Blumberg *et al.*, 1989). There is also evidence that HR may protect vascular endothelium against oxidative injury (Nees, 1992).

In view of these findings, we were interested in whether flavonoids, in particular HR, are able to protect myocardium against oxidative damage. Therefore, the present *in vitro* study was carried out to examine the effect of HR on singlet oxygen challenged isolated cardiomyocytes of the adult rat.

Methods

Isolation of cardiomyocytes

Single cardiomyocytes were isolated from adult rat hearts according to the method described by Piper *et al.* (1982) with some minor modifications as described previously (Ver Donck *et al.*, 1986). Hearts of male Wistar rats (body weight ranging from 250-300 g) were isolated and perfused via the aorta by an oxygenated modified Krebs-Ringer-HEPES (KRH) buffer (pH 7.3, 37°C, flow rate: 10 ml min^{-1}) of the following composition (mM): NaCl 125, KCl 2.6, KH_2PO_4 1.2, $MgSO_4 \cdot 7\ H_2O$ 1.2, glucose 5.5 and HEPES 10. After 5 min perfusion, the hearts were perfused for another 25 min with a KRH buffer supplemented with 25 μM $CaCl_2$, 0.06% collagenase (Wako, Neuss, Germany), and 0.1% fatty acid-free bovine serum albumin (BSA) (Sigma Chemie, Deisenhofen, Germany). After dispersion of the cells, and a stepwise increase of $CaCl_2$ to 0.5 mM, aliquots of the cell suspension were layered on a 10 cm high column with a KRH buffer containing 2% BSA (Serva Feinbiochemica, Heidelberg, Germany) and a final concentration of 1 mM $CaCl_2$. Myocytes were allowed to settle for 10 min and the supernatant was removed. The remaining cell suspension was resuspended with KRH buffer (supplemented with 1 mM $CaCl_2$, pH 7.4) and aliquots were divided into plastic test tubes. The protein content for the myocyte

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suspension was determined according to the method described by Lowry *et al.* (1951). All experiments were carried out at room temperature (20°C).

Reactive oxygen species

Reactive oxygen species were generated by photo-excitation of the light sensitive dye rose bengal producing singlet oxygen as described previously by Ver Donck *et al.* (1988). Thirty minutes prior to photo-excitation rose bengal (10^{-7} M) alone or in combination with HR was added to the KRH incubation medium. A fibreoptic connected to a cold light source (150 W Xenophot lamp KL 1500, Schott, Mainz, Germany) was positioned in the test tube 2 cm above the surface of the cell suspension, and the tube was wrapped with a reflecting foil. The cell suspension was then illuminated for a fixed period of 80 or 120 s. For the determination of the number of rod shaped cells and of thiobarbituric acid reactive substances (TBARS) formation aliquots of the suspension were removed immediately before and 15 min after termination of the illumination.

Cell count and morphological evaluation

About 90% of the cardiomyocytes in the cell suspension in KRH displayed a rod shaped morphology. The number of calcium tolerant rod shaped cardiomyocytes was determined by evaluating aliquots of the cell suspension with an improved Neubauer counting chamber (Fischer, Frankfurt am Main, Germany). For this purpose five squares of the Neubauer counting chamber were evaluated at a magnification of × 100, and the number of rod shaped cells (around 50 cells) was counted. The volume of one square amounts to 0.1 µl. Therefore, the number of rod shaped cells per ml suspension amounts to: counted cells $\times 2 \times 10^3 =$ cells ml^{-1}.

Singlet oxygen-induced cell damage was assessed on the basis of shape changes from elongated rod shaped to hypercontracted rounded cells (Figure 1). Such shape changes of isolated cardiomyocytes are regarded to result from intolerance to physiological calcium concentrations due to membrane injury (Slade *et al.*, 1983).

Assessment of lipid peroxidation

Accumulation of secondary lipid peroxidation products reacting with thiobarbituric acid was measured spectrophotometrically according to a method described by Wong *et al.* (1987). Plasma lipoperoxides were hydrolysed by boiling in dilute phosphoric acid (0.44 M H_3PO_4). The hydrolysis products consisting of malondialdehyde (MDA) and other aldehydes were reacted with thiobarbituric acid (42 mM) to form thiobarbituric acid reactive substances (TBARS). Plasma proteins were precipitated with methanol-NaOH (0.09 M) and removed from the medium by centrifugation (9.500 × *g*). The amount of thiobarbituric acid reactive substances was then quantified spectrophotometrically by the absorbance at 532 nm. Lipoperoxide concentrations were calculated by reference to a calibration curve prepared by means of tetramethoxypropan (TMP) which liberates stoichiometric amounts of MDA when hydrolysed in the presence of thiobarbituric acid (Gutteridge, 1975).

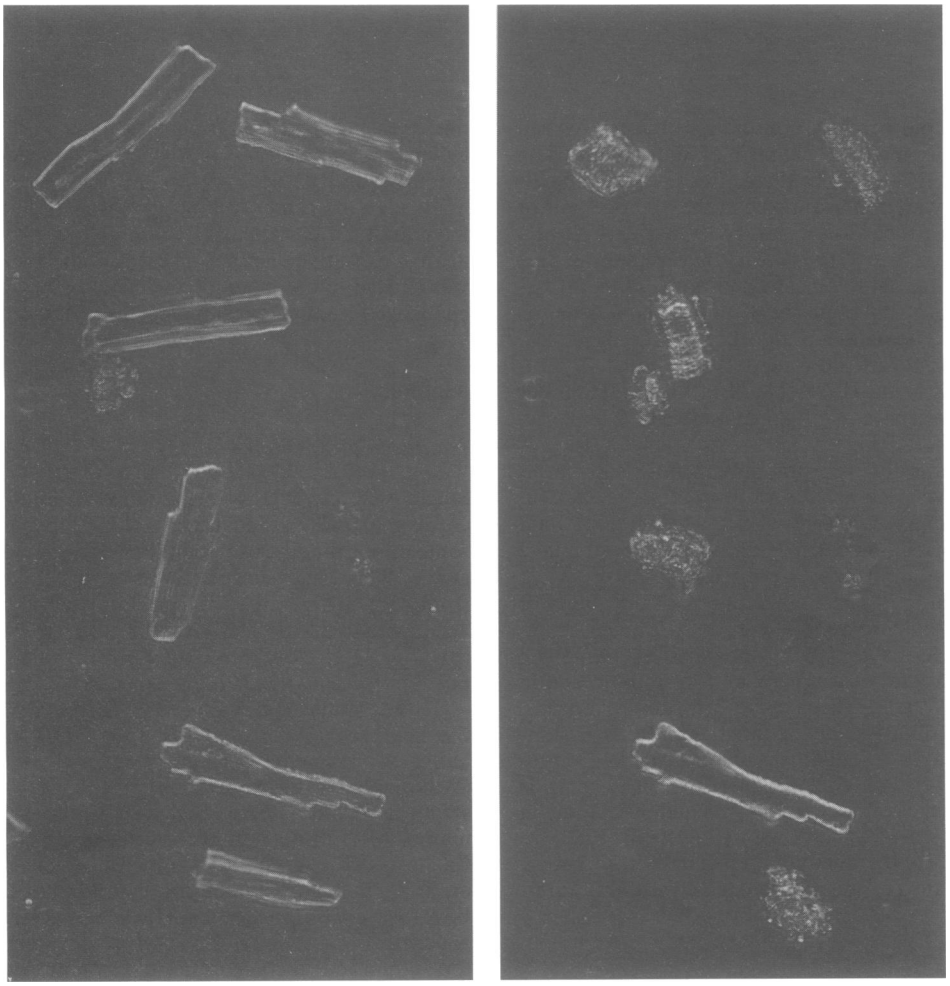

Figure 1 Rose bengal/560 nm light-induced shape changes of cardiomyocytes from elongated rod shaped to hypercontracted rounded cells. (Left) Before illumination, (right) 15 min after illumination (80 s), magnification × 400.

Experimental protocol

To investigate the effect of HR on rose bengal/light-induced cell damage each analytical run for morphological evaluation of the cardiomyocytes and determination of TBARS consisted of duplicate assays of the following probes: untreated cells, cells incubated with rose bengal for 30 min in dark, cells incubated with rose bengal 15 min after illumination, cells incubated with rose bengal and HR for 30 min in dark, cells incubated with rose bengal and HR 15 min after illumination. In the case of the TBARS determination each experiment included the duplicate assay of TMP working standard solutions. According to this protocol the examination of one drug concentration afforded one separate preparation. In an additional set of experiments, the effect of HR alone on the morphology of isolated cardiomyocytes was tested.

Drugs

Rose-bengal (Sigma Chemie, Deisenhofen, Germany) was dissolved in KRH-buffer with 1 mM $CaCl_2$ at a concentration of 10^{-3} M and further diluted to its final concentration in the same buffer. A standardized mixture of HR (by courtesy of Zyma, München, Germany) was also dissolved in KRH-buffer with 1 mM $CaCl_2$ at a concentration of 67 mg 100 ml^{-1} and further diluted to its final concentration in the same buffer. HR is comprised mainly of monohydroxyethylrutosides (about 5%), dihydroxyethylrutosides (about 34%), trihydroxyethylrutosides (about 46%), and tetrahydroethylrutosides (about 5%), as determined by high performance liquid chromatography (specification 0-[β-hydroxyethyl]-rutosides, unpublished data on file, Zyma, Munich, Germany).

Statistical analysis

Each measurement was done in duplicate. The data are presented as the mean ± s.e.mean of n separate preparations (animals). Statistical analysis of the data was carried out by employing analysis of variance and the two-sided Mann-Whitney-U-test (Sachs, 1992). A P value <0.05 was considered to be statistically significant.

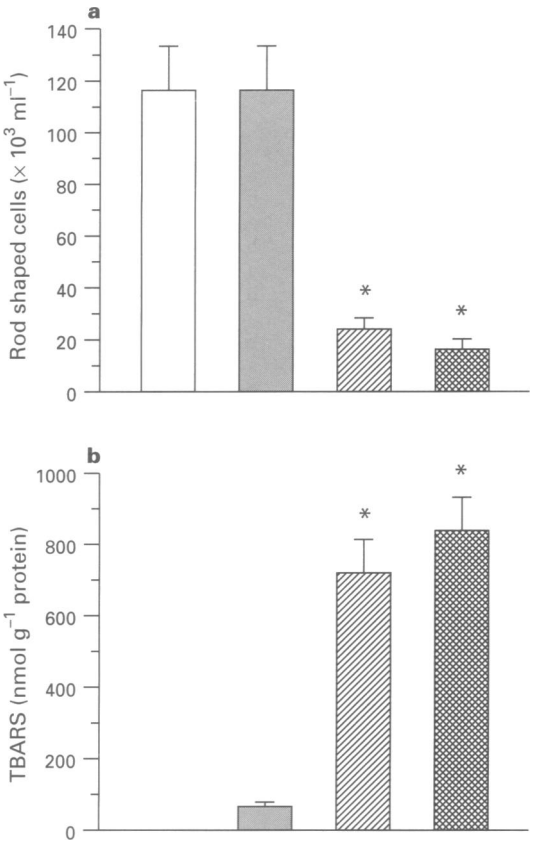

Figure 2 The effect of rose bengal/light on the number of rod shaped cardiomyocytes (a) and formation of thiobarbituric acid reactive substances (TBARS) (b). (Open columns): after isolation procedure; (stippled columns), during incubation with rose bengal (10^{-7} M) in dark; (hatched columns), after 80 s illumination; (cross-hatched columns), after 120 s illumination; $n=6$ separate preparations. *$P<0.005$ vs. incubation with rose bengal in dark.

Results

Effect of photo-excitation of rose bengal

Photo-excitation of rose bengal (10^{-7} M) resulted in a sharp and rapid loss of rod shaped cells (Figure 1) and a 12 fold increase in the production of thiobarbituric acid reactive substances indicating lipid peroxidation (Figure 2). Both effects slightly depended on the duration of illumination, i.e. they were more pronounced after 120 s as compared to 80 s of illumination. In the experiments in which HR were used in combination with rose bengal a fixed exposure time of 80 s was chosen.

Control experiments

After addition of rose bengal (10^{-7} M) to the cell suspension followed by 30 min of incubation in the dark the number of rod shaped cells remained constant (Figure 2). Illumination of the cardiomyocyte suspension in the absence of rose bengal did not influence the number of rod shaped cells either (data not shown).

Effect of HR

Since the cardiomyocytes displayed a slight variability in sensitivity to rose bengal/light in spite of using a fixed exposure time to illumination each experiment included a control consisting of sole incubation with rose bengal. Co-incubation with HR in addition to rose bengal increased the number of surviving rod shaped myocytes after photo-excitation in a dose-dependent bell-shaped manner suggesting a protective action against oxidative damage. The effect proved to be significant with concentrations of HR ranging from 67 pg ml^{-1} to 67 ng ml^{-1} (Table 1, Figure 3). Lower and higher concentrations failed to improve the number of rod shaped cells although a slight tendency for a protective effect appeared at 670 μg ml^{-1} which was the highest concentration of HR investigated in these experiments (Figure 3).

Rutosides like other flavonoids are regarded to possess antioxidative activity. In order to assess whether the protective effect of HR was due to a reduced lipid peroxidation, the extent of TBARS production as an indicator for lipid peroxidation was measured. Despite a significant protection against singlet oxygen induced hypercontracture of the cells HR did not affect the high amount of TBARS production after exposure to the singlet oxygen generating system as compared to the controls (Table 1, Figure 4). A significant reduction in the amount of TBARS production after photo-excitation of rose bengal associated with the aforementioned slight but not significant improvement in the amount of rod shaped cells only occurred at the highest HR concentration of 670 μg ml^{-1} (Figure 3).

In order to exclude a possible toxic action of HR *per se*, especially at concentrations above 67 ng ml^{-1}, cardiomyocytes were incubated with rutosides alone and illuminated. At neither concentration did HR show any effect on the myocytes *per se* as compared to incubation with the buffer alone (Table 2).

Table 1 Effect of β-hydroxyethyl rutosides (HR) on the number of rod shaped cells and content of thiobarbituric acid reactive substances (TBARS) in suspensions of cardiomyocytes after exposure to rose bengal/light

		Rose bengal alone		*Rose bengal + HR*		
		Rod shaped cells	*TBARS*	*Rod shaped cells*	*TBARS*	
HR		(cells × 10^3 ml^{-1})	(nmol g^{-1} protein)	(cells × 10^3 ml^{-1})	(nmol g^{-1} protein)	n
6.7 pg ml^{-1}	a	119.0±19.61	32.2±26.2	124.17±19.14	28.2±11.2	6
	b	11.67±2.22	473.2±95.3	11.67±2.32	568.8±145.0	
67 pg ml^{-1}	a	103.33±17.31	29.2±12.4	96.33±14.76	34.6±21.5	6
	b	11.0±3.75	539.5±73.3	28.67±15.75*	445.1±42.9	
670 pg ml^{-1}	a	112.0±29.83	23.8±12.1	112.83±28.03	15.4±11.4	6
	b	24.0±10.08	287.4±69.0	68.0±22.24*	353.4±73.4	
6.7 ng ml^{-1}	a	54.0±9.18	10.5±3.4	57.17±9.66	14.2±9.9	6
	b	7.0±1.69	236.0±17.7	20.0±2.99*	203.1±24.5	
67 ng ml^{-1}	a	136.5±16.41	36.6±11.1	129.75±17.36	41.9±17.9	7
	b	42.38±10.27	298.4±80.0	76.75±12.46*	299.5±73.4	
670 ng ml^{-1}	a	87.33±13.16	26.9±6.6	89.17±16.33	37.9±13.3	6
	b	34.33±11.31	239.4±34.70	36.17±10.91	274.5±59.6	
6.7 μg ml^{-1}	a	58.33±2.33	26.7±16.9	57.33±3.22	14.4±7.0	6
	b	10.17±4.61	424.4±37.0	11.17±4.74	440.5±40.9	
67 μg ml^{-1}	a	46.8±8.75	31.2±10.9	46.2±8.18	16.8±3.1	5
	b	10.4±4.4	297.3±78.5	11.6±5.26	364.8±79.7	
670 μg ml^{-1}	a	60.4±9.37	29.9±6.3	65.2±11.3	37.5±14.2	5
	b	9.8±5.62	424.7±97.5	16.2±8.3	309.1±86.8¶	

Data shown are mean±s.e.mean; n = number of separate preparations. a = incubation in dark, b = after illumination. *$P<0.05$ vs. rose bengal alone, ¶$P=0.059$ vs. rose bengal alone.

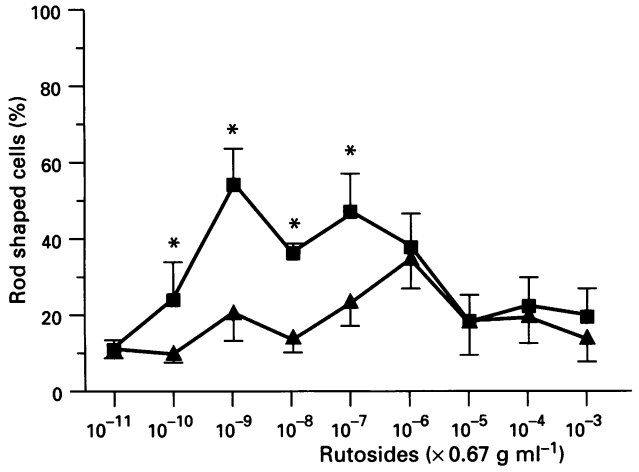

Figure 3 The effect of β-hydroxyethyl rutosides (HR) on singlet oxygen-induced loss of rod shaped cardiomyocytes (due to hypercontracture). Singlet oxygen was generated by illumination of rose bengal (see Methods). Percentage of rod shaped cells 15 min after illumination (80 s) in the presence of rose bengal (10^{-7} M) alone (▲) or in combination with various concentrations of HR (■); $n=5$ to 7 separate preparations. *$P<0.05$ vs. rose bengal alone.

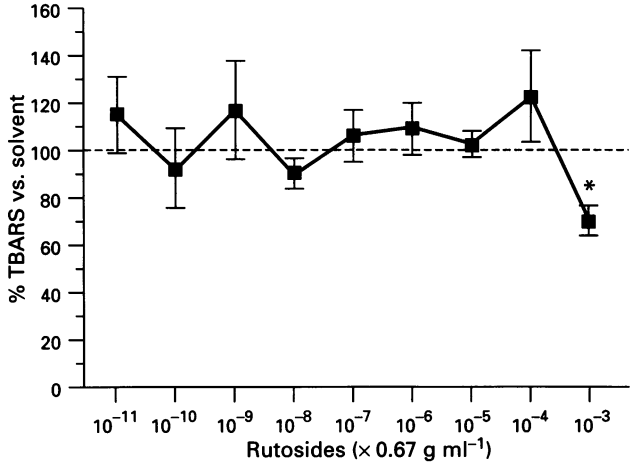

Figure 4 Relative amount of thiobarbituric acid reactive substances (TBARS) in the presence of β-hydroxyethyl rutosides (HR; 6.7 pg ml^{-1} to 670 μg ml^{-1}), in addition to rose bengal, in relation to incubation with rose bengal alone (=100%). $n=5$ to 7 separate preparations. *$P=0.059$ vs. rose bengal alone.

Discussion

In the present study, the effect of HR on singlet oxygen challenged isolated cardiomyocytes was examined. The value of the isolated heart muscle cell for structural and functional studies has widely been recognized for more than ten years (Dow *et al.*, 1981). Advantages of single cell preparations are the homogeneity of the cell population which makes it possible to evaluate the characteristics of individual cells in the absence of external influences, i.e. interactions between myocytes and microvasculature, regional differences in blood flow, influences of neighbouring tissues, neuronal, mechanical, and hormonal modulation (Dow *et al.*, 1981; Piper *et al.*, 1982; Borgers *et al.*, 1988). In addition, since a large number of cells are obtained from one organ, a multitude of experiments can be carried out, thereby saving on experimental animals. On the other hand, experimental methods with isolated cells are not devoid of shortcomings (Borgers *et al.*, 1988). The proteolytic enzymes used during isolation may have altered some of the sarcolemmal components and may therefore influence certain cell functions. Also, removing cells from their natural habitat creates experimental conditions which may profoundly differ from the *in vivo* situation, e.g. the bathing medium of isolated cells represents an infinite space in contrast to the limited extracellular space in intact myocardium. This may result in extra- and intracellular concentrations of ions and metabolites which differ from those found in multicellular preparations. Therefore, discrepancies between *in vivo* and *in vitro* situations are inevitable; however, they do not necessarily invalidate the model.

The data presented in this *in vitro* study show that HR can protect isolated cardiac myocytes against singlet oxygen-

Table 2 Percentage of rod shaped cells after 30 min of incubation with β-hydroxyethyl rutosides (HR) alone

Concentration of HR	*Rod shaped cells* (%)	n
HR free KRH-buffer	88.7±3.4	4
6.7 pg ml^{-1}	89.9	2
67 pg ml^{-1}	90.5	2
670 pg ml^{-1}	93.4	2
6.7 ng ml^{-1}	95.3	2
67 ng ml^{-1}	99.3	2
670 ng ml^{-1}	87.6	2
6.7 μg ml^{-1}	87.0±4.7	4
67 μg ml^{-1}	84.1±5.3	4
670 μg ml^{-1}	92.0±4.6	4

Data shown are means±s.e.mean if *n* (number of separate preparations) > 2.

induced damage. Singlet molecular oxygen was produced by photo-excitation of the light sensitive dye rose bengal (Pooler & Valenzo, 1981; Gandin *et al.*, 1983; Ver Donck *et al.*, 1988). In aqueous solution, rose bengal can be elevated to a triplet state by illumination with light (500–600 nm) and, in the presence of oxygen, this triplet decays producing mainly singlet oxygen (Lee & Rogers, 1987). This reactive oxygen species forms endoperoxides in unsaturated lipids by additional reactions with isolated double bonds (Rawls & Van Santen, 1970; Halliwel & Gutteridge, 1985). *In vivo* singlet oxygen may be directly involved in the 'oxygen paradox' (Gauduel & Duvelleroy, 1984). The deleterious effect of rose bengal/photo-excitation on isolated cardiac myocytes, i.e. shape changes from rod shaped to hypercontracted rounded cells, associated with the accumulation of TBARS indicative of lipid peroxidation in the present study is consistent with previous findings from Ver Donck *et al.* (1988).

The effect of HR on oxidative damage of cardiomyocytes has been studied over a wide concentration range. The protective action occurred at comparatively low (nanomolar) concentrations and displayed a bell-shaped dose-response curve. This finding confirms a preliminary observation from our laboratory (Olbrich *et al.*, 1994). Interestingly, HR caused an improvement of the number of elongated rod shaped cardiomyocytes during exposure to singlet oxygen without reducing the accumulation of TBARS. This suggests that in this *in vitro* model HR might not have acted as a scavenger for reactive oxygen species. In contrast, in a previous study rutin, at submillimolar (10^{-4} M) concentrations, was shown to act as a scavenger for singlet oxygen and to suppress lipid peroxidation (Sorata *et al.*, 1984). In other studies concerning the vasoprotective action of rutosides and related flavonoids their protective activity was also attributed to scavenging of free radicals (Gryglewski *et al.*, 1987; Robak & Gryglewski, 1988). The discrepancy with our present findings might be explained in part by the considerably lower effective concentrations in the present study as compared to others (Sorata *et al.*, 1984; Robak & Gryglewski, 1988). At high concentrations of HR (670 μg ml^{-1}) we also observed a significant reduction of TBARS accumulation which was associated with a slight improvement in the number of intact cardiomyocytes.

There are only a few studies dealing with the effects of flavonoids on myocardium. Recent experimental studies with Langendorff perfused isolated guinea-pig hearts revealed an increased coronary flow and relaxation velocity caused by flavonoids including rutin (Schüssler *et al.*, 1995); these effects were attributed to inhibition of adenosine 3',5'-cyclic monophosphate (cyclic AMP) phosphodiesterase. HR also has been shown to be able to protect myocardium against the toxicity of antitumour agents of the anthracyclin-type (Van Acker *et al.*, 1995). Although in those studies no parameters of lipid peroxidation were measured, the authors attributed the protective effects to the antioxidative activities of HR.

However, the mechanism of action and the reason for the bell-shaped dose-response curve are not clear. The protection could have been due to absorption of light by HR responsible for activation of rose bengal. This seems unlikely because (1) the production of TBARS was not influenced by protective concentrations, (2) the protection ceased at higher concentrations, and (3) the maximum light absorption for HR lies below 400 nm (data not shown). The lack of protection at high rutoside concentrations could be due to a dose-dependent toxicity of rutoside itself. However, incubation with HR covering the whole concentration range in the absence of rose bengal had no negative effect on the cardiomyocytes, leaving this possibility unlikely. A bell-shaped dose-response curve for HR has also been described recently in experimental studies on the permeability of microvessels in the frog mesentery (Kendall *et al.*, 1993), as well as in clinical trials in patients with chronic venous insufficiency (Diebschlag *et al.*, 1994). Flavonols have been shown to bind to biomembranes (Sorata *et al.*, 1984) especially to membrane proteins (Gryglewski *et al.*, 1987) which may result in the prevention of destabilization of cellular membranes. To explain the bell-shaped dose-response relationship Kendall *et al.* (1993) offered the hypothesis that HR bind at two different sites which give rise to opposite effects. According to Young *et al.* (1992) a bell-shaped dose-response curve indicates the involvement of separate binding sites exerting non-competitive antagonism. With regard to the present data, a bell-shaped dose-response would be expected if the affinity of HR for the binding site that promotes membrane stabilization is higher than that which leads to destabilization.

We do not know how the concentrations of HR used here relate to clinically attainable concentrations. Data on the absorption and metabolism of flavonoids are scarce and inconclusive (Gugler *et al.*, 1975; Ueno *et al.*, 1983). According to Hackett *et al.* (1976) HR is absorbed after oral administration in man and disappears rapidly from the blood. The recommended oral dose of 500 mg daily has proven to be effective in reducing oedema in post-thrombotic syndrome (Diebschlag *et al.*, 1994). In experimental studies HR reduced microvascular permeability at concentrations equal or greater than 10 μg ml^{-1} (Kendall *et al.*, 1993). In view of these data it appears reasonable to assume that the concentrations used in the present investigation may be relevant to the clinical situation.

The authors wish to thank Mrs B. Henke for her expert technical assistance.

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(Received December 1, 1995
Revised May 13, 1996
Accepted July 19, 1996)