Free Rad. Res. Comms., Vol. 1, No. 3, pp. 225–233
8755-0199/86/0103-0225 $25.00/0
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Printed in Great Britain

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THE EFFECT OF VITAMIN E-DEFICIENCY IN ISOLATED RAT HEART ON THE CELLULAR DEFENCE SYSTEM AGAINST FREE RADICALS DURING NORMAL REPERFUSION AFTER HYPOXIC, ISCHEMIC AND Ca^{2+}-FREE PERFUSION

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(Received May 20, 1985)

We investigated whether vitamin E plays a role in the protection against potential free radical formation and related biochemical changes in hypoxic, ischemic and Ca^{2+}-depleted rat heart upon normal reperfusion.

In the heart of normally fed rats a decrease in the activity of superoxide dismutase and the capacity of the glutathione system, factors of the cellular protective mechanisms against free radicals, occurred upon exposure to the above mentioned treatments. This decrease was not further enhanced if vitamin E-deficient rat hearts were treated. Vitamin E-deficiency, however, led to detectable peroxidation of lipids if Ca^{2+}-depleted or hypoxic hearts were reperfused. Lipid peroxidation was measured as the formation of thiobarbituric acid reactive material, which is readily formed during this process. Reflow after ischemia did not induce lipid peroxidation either in normal or in vitamin E-deficient rat heart.

Since changes in Ca^{2+}-homeostasis are thought to be primarily responsible for the Ca^{2+}-reperfusion injury, a role for Ca^{2+}-ions in lipid peroxidative processes, either directly or indirectly, seems indicated. Furthermore the results imply that even a sharp and extensive decrease of reduced glutathione, as seen upon Ca^{2+}-repletion after a period of Ca^{2+}-depletion, does not necessarily induce peroxidation of lipids in heart tissue. Obviously, vitamin E is very important in the protection of cardiac membranes. Replenishment of the water-soluble protective factors in the heart seems, however, more important during above mentioned treatments, especially since repair of the vitamin E-free radical is dependent on water-soluble factors.

Key words: ischemia; hypoxia; Ca^{2+}-paradox; lipid peroxidation; vitamin E; isolated rat heart

INTRODUCTION

Excessive formation of free radicals may play an important role in the pathogenesis of myocardial damage which develops after a period of hypoxia or ischemia[1,2,3]. Upon reoxygenation of previously hypoxic or ischemic tissue an exacerbation of free radical formation is hypothesized to occur[4]. Under physiological conditions a continuous production of free radicals occurs, e.g. during electron transport in the mito-

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chondria[5]. The superoxide anion radical ($O_2^{\cdot -}$), the major reactive species formed in mitochondria due to an one electron reduction of oxygen, is converted to H_2O_2 by superoxide dismutase (SOD). Because of the low catalase activity in heart cells, this H_2O_2 is then converted into H_2O by glutathione-peroxidase (GSH-Px). The latter reaction needs glutathione (GSH) as a substrate. The oxidized form of GSH, GSSG, is reduced again by glutathione-reductase. The capacity of these cellular defence mechanisms against free radicals decreases during normal reperfusion of rat heart after hypoxic or ischemic treatment as we previously reported[6]. Peroxidation of lipids, which is thought to occur readily when formation of reactive intermediates is enhanced in tissue, was however not found. In case hearts were repleted with Ca^{2+} after a period of Ca^{2+}-free perfusion, the so-called Ca^{2+}-paradox phenomenon, an even more significant decrease in the capacity of the factors of the cellular protective mechanism, was observed. Recently it was suggested that sudden changes in the concentration of Ca^{2+}, which are thought to be primarily responsible for the Ca^{2+}-reperfusion injury during the Ca^{2+}-paradox[7,8], disturb the balance between the generation of reactive substances on the one hand and the protection against free radicals on the other hand[9,10]. However, no formation of thiobarbituric acid reactive material (TBA-rm), which is formed during lipid peroxidation, was found during this treatment[6]. We investigated whether vitamin E, an important lipid-soluble antioxidant[11], plays a role in the prevention of lipid peroxidation during normal reperfusion after hypoxic, ischemic or Ca^{2+}-free perfusion of isolated rat heart.

The results show that vitamin E is decisive in the protection of membrane lipids if the heart is repleted with O_2 or Ca^{2+}-ions after a period of depletion. It cannot, however, prevent attack on the other defence mechanisms.

MATERIALS AND METHODS

GSH-reductase (type III), cytochrome c_{oxid} (from horse heart, type III), SOD (from bovine blood, type I), xanthine oxidase (from buttermilk, grade I) and β-NADPH were purchased from Sigma, St. Louis, USA. 2-Vinylpyridine was obtained from Merck, Darmstadt, W-Germany and stored under nitrogen at $-20°$ C after purification by vacuum distillation. All other chemicals were of analytical grade purity.

The control rats (male, Wistar) were fed a semi-synthetic stock diet (Muracon SSP-Tox standard, Trouw NV, Putten, The Netherlands) for 3 months. The vitamin E-deficient rats were fed the same diet, from which vitamin E, however, was omitted.

Perfusion method

Rats of either group were anesthesized with diethylether. After heparinization of the rat, the aorta was cannulated, the heart quickly removed and perfused according to the method of Langendorff[12]. The standard salt solution contained (mM): NaCl 128.2; KCl 4.7; $CaCl_2$ 1.4; $MgCl_2$ 1.1; NaH_2PO_4 0.4; $NaHCO_3$ 20.2; glucose 11.1 and was continuously gassed with 95% O_2-5% CO_2 (pH 7.4; 37° C). During hypoxic perfusion the standard salt solution was continuously gassed with 95% N_2-5% CO_2, providing a very low PO_2. In order to obtain a Ca^{2+}-free perfusion solution, the $CaCl_2$ was omitted from the standard salt solution. The hearts were paced at 300 beats/min and stabilized for 30 mins. at a constant pressure of 8 kPa and 10 min at a constant flow of 6 ml/min, which resulted in a pressure of 8 ± 2 kPa. After either a hypoxic

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perfusion of 60 min, or a Ca^{2+}-free perfusion of 10 min — both at a flow of 6 ml/min — or a 1 hour period of flow stop to create total ischemia, a recirculating, constant flow perfusion of 6 ml/min was started using 25 ml of the standard salt solution. At the indicated sample-times 2 ml of the perfusate were collected. The heart tissue was rapidly frozen at the end of the perfusion using Wollenberger clamps precooled in liquid nitrogen[13]. Recently it has been reported that mannitol, which is often used to replace glucose during hypoxic perfusion of cardiac tissue, provided protection to the myocardium as a result of its ability to scavenge hydroxyl radicals[14]. Glucose, which is used in our investigations, is a good scavenger of hydroxyl radicals as well[15]. It must therefore be borne in mind that compounds like these can mask free radical formation in the myocardial tissue to some extent.

Biochemical assays

The hearts were homogenized with a Polytron PT-10 in an ice-cold 50 mM phosphate buffer, pH 7.4 containing 0.1 mM EDTA. The vitamin E content of the homogenate was extracted according to Rammel et al.[16], using HPLC with fluorimetric detection[17]. The vitamin E content in hearts of vitamin E-deficient rats amounted to 7–13% of the vitamin E-content in the hearts of rats which were fed the normal stock diet. The GSH-Px activity was assayed in the homogenate according to the method of Lawrence and Burk[18]. The activity of the Se-dependent GSH-Px was monitored at 340 nm by the disappearance of NADPH, using hydrogen peroxide as a substrate. The amount of GSH and GSSG in homogenate and coronary effluent were determined as described by Griffith[19]. According to this method the amount of GSSG was measured after binding the GSH by 2-vinylpyridine. The procedure of Weisiger and Fridovich[20], using the cytochrome c/xanthine oxidase system, served as a method for the determination of SOD. Fatty acid composition of heart lipids were determined by gas chromatography on a fused silica column (CP tm Sil 88, Chrompack, Middel-

TABLE I

The activity or content of important factors in this study, as measured in the cardiac tissue of rats fed a complete or vitamin E-deficient diet. The content of polyunsaturated fatty acids (PUFA — 18:2, 20:4 and 22:6) is expressed as a fraction of the total amount of fatty acids (TFA — 16:0, 18:0, 18:1, 18:2, 20:4 and 22:6). Measurements were performed in perfused, untreated tissue.

DIET	COMPLETE DIET	VITAMIN E-DEFICIENT
VITAMIN E (*ng/mg protein*)	197 ± 19	19 ± 4
SOD (*U/mg protein*)	13.8 ± 1.1	14.1 ± 1.7
GSH-Px (Se-dependent) (*nmol NADPH/min/mg protein*)	88 ± 4	83 ± 3
GSH + GSSG (*nmol GSH-eq./mg protein*)	14.6 ± 0.7	13.8 ± 0.3
TBA-rm (*nmol/mg protein*)	0.025 ± 0.003	0.023 ± 0.005
$\frac{PUFA}{TFA}$	0.41 ± 0.02	0.40 ± 0.02

Each result represents the mean ± SEM of 6 rats. No statistically significant difference was found between these parameters in cardiac tissue of either group of rats.

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burg, The Netherlands) after extraction, saponification and methylation according to Folch et al.[21]. The amount of lipid peroxidation was assayed in heart homogenate and perfusate as thiobarbituric acid reactive material, according to the method of Buege and Aust[22]. Lipid peroxidation was expressed as nmol MDA using an extinction coefficient of $1.56.10^5\ M^{-1}\ cm^{-1}$ at 535–600 nm.

Statistics

The data presented are expressed as a mean ± SEM and were statistically evaluated by the Student's t-test.

RESULTS

Omission of vitamin E from the stock diet might influence the content and/or activity of many cell constituents that are important in this investigation. Therefore we mea-

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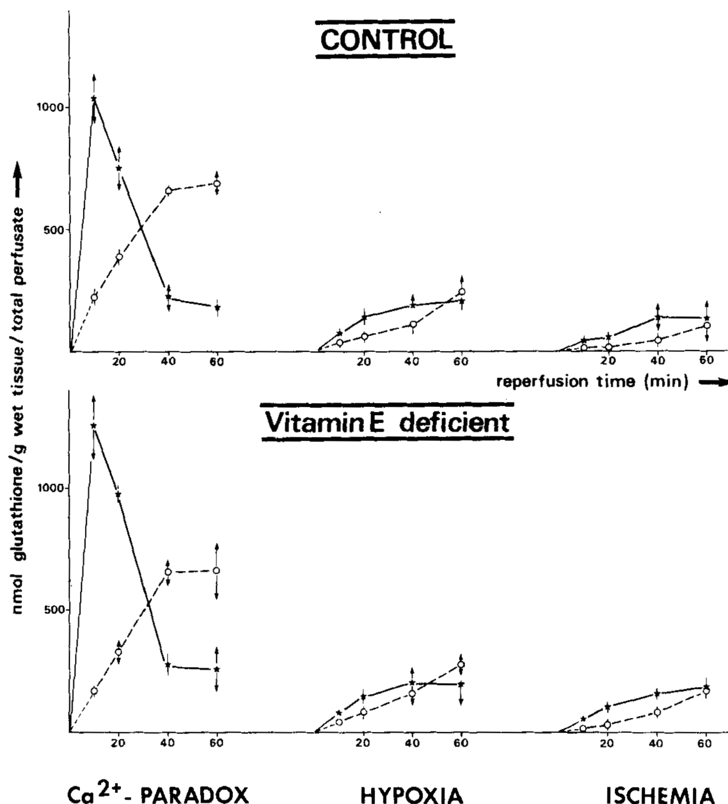

FIGURE 1 The cumulative release of GSH (—*—) and GSSG (--o--) into the recirculated perfusate of control and vitamin E-deficient rat heart, measured upon normal reperfusion during 60 minutes after a Ca^{2+}-free, hypoxic or ischemic period. Each value represents the mean ± SEM of at least 4 separate experiments. None of the treatments introduced a difference between control and vitamin E-deficient hearts that was significant according to the Student's t-test ($p > 0.05$).

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sured the intrinsic values of the factors of the cellular protective system, SOD and the constituents of the glutathione system, and the amount of fatty acids in the cardiac tissue of rats fed a complete or a vitamin E-deficient diet. As Table I shows no differences were observed between both groups of rats, as far as these parameters in heart tissue were concerned.

Figure 1 depicts the cumulative release of GSH and GSSG into the coronary effluent of both control and vitamin E-deficient rat heart during normal reperfusion after a period of Ca^{2+}-free, hypoxic and ischemic perfusion. The low content of vitamin E in vitamin E-deficient rate heart did not cause a different release pattern of GSH and GSSG into the perfusate compared to control hearts. In addition no

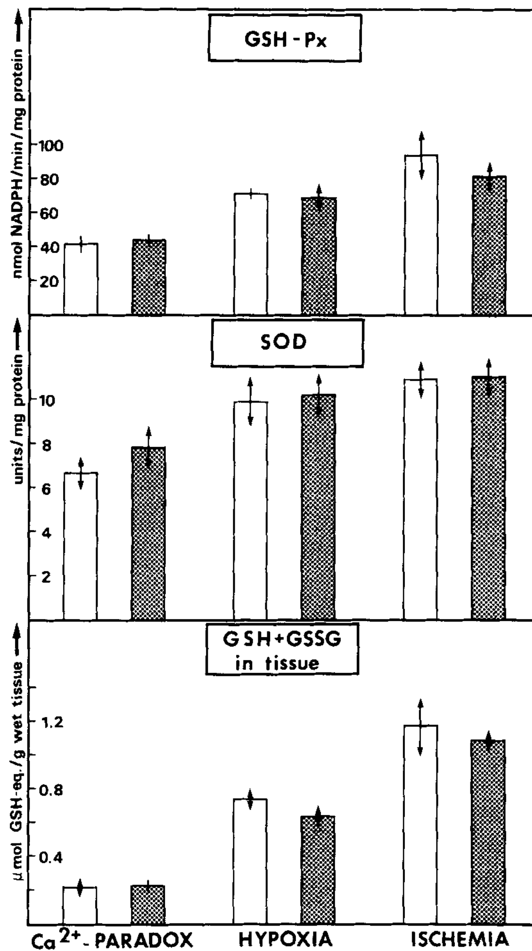

FIGURE 2 The effect of a recirculating normal reperfusion during 60 minutes after a Ca^{2+}-free, hypoxic or ischemic treatment, on the activity of SOD, GSH-Px and total glutathione content in control □ or vitamin E-deficient □ rat heart. Each result is the mean ± SEM of at least 4 separate experiments. None of the treatments introduced a significant difference between control or vitamin E-deficient rat hearts ($p > 0.05$).

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TABLE II
The content of TBA-rm in heart tissue of rats fed a complete or vitamin E-deficient diet upon a recirculating, normal reperfusion for 60 minutes after a Ca^{2+}-free, hypoxic or ischemic treatment.

TREATMENT followed by normal reperfusion	TBA-rm in heart tissue (*expressed as nmol MDA/g wet tissue*)	
	CONTROL	VITAMIN E-DEFICIENT
Ca^{2+}-free	7.5 ± 0.8	14.4 ± 0.4**
Hypoxic	5.8 ± 0.7	9.5 ± 0.9*
Ischemic	5.2 ± 0.7	6.3 ± 0.4

Each value represents the mean of at least 4 separate experiments. Significance of difference between control (E+) and vitamin E-deficient (E−) heart tissue: * $p < 0.01$; ** $p < 0.001$. 1 Gram wet tissue contained (mg protein): Ca^{2+}-free 140 ± 4 (E+), 131 ± 6 (E−); hypoxic 145 ± 3 (E+), 137 ± 5 (E−); ischemic 158 ± 2 (E+), 142 ± 3 (E−).

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increased formation of GSSG was observed during the above mentioned treatments. The release of GSH was not significantly faster in vitamin E-deficient rat hearts.

As reported before[6], normal reperfusion of Ca^{2+}-depleted, hypoxic or ischemic rat hearts decreases the cellular defence mechanisms against free radicals. Due to vitamin E-deficiency, the decrease in the activity of SOD and GSH-Px in tissue was not further enhanced (Figure 2). The total amount of glutathione, which was left in the vitamin E-deficient hearts after the treatments, was not further diminished as well.

In the coronary effluent of control hearts exposed to a recirculating reperfusion after a Ca^{2+}-free, hypoxic or ischemic period, no formation of TBA-rm could be detected (Figure 3). However, in the perfusate from vitamin E-deficient rat hearts a time-dependent increase in formation of TBA-rm was found upon reperfusion after a Ca^{2+}-free or hypoxic period (Figure 3). An increased content of TBA-rm was also measured in the heart tissue of vitamin E-deficient rats upon Ca^{2+}-repletion after a Ca^{2+}-free period or reoxygenation after hypoxia (Table II). Vitamin E-deficiency did not result in an increased content of TBA-rm in the heart tissue (Table II) or release into the perfusate (Figure 3) upon normal reperfusion after a period of ischemia.

DISCUSSION

There is a rapidly accumulating body of evidence that free radicals, in particular reactive oxygen species, play a substantial role in the induction of cardiac damage upon reperfusion of hypoxic or ischemic hearts[23,24]. Due to inhibition of the terminal component of the respiratory chain in the mitochondria, an accumulation of reduced electron carriers occurs. Upon reoxygenation or reflow a burst in the formation of activated oxygen species can be expected. Possibly an incomplete reduction of the molecular oxygen dissolved in the matrix takes already place during the ischemic or hypoxic period. In addition a high increase of $O_2^{\bar{}}$ production has been associated with the conversion of hypoxanthine to xanthine by the enzyme xanthine oxidase with the reintroduction of oxygen. The amount of hypoxanthine in the heart is increased due to ATP-degradation during ischemia[25,26]. Recently it has also been reported that sudden changes in Ca^{2+}-concentrations led to lipid peroxidation in erythrocytes[9]. Data obtained from experiments with heart mitochondria and microsomes also pointed at a relationship between alterations of the Ca^{2+}-concentration and activation of lipid peroxidation[10]. A disturbance of the normal intracellular Ca^{2+}-homeostasis,

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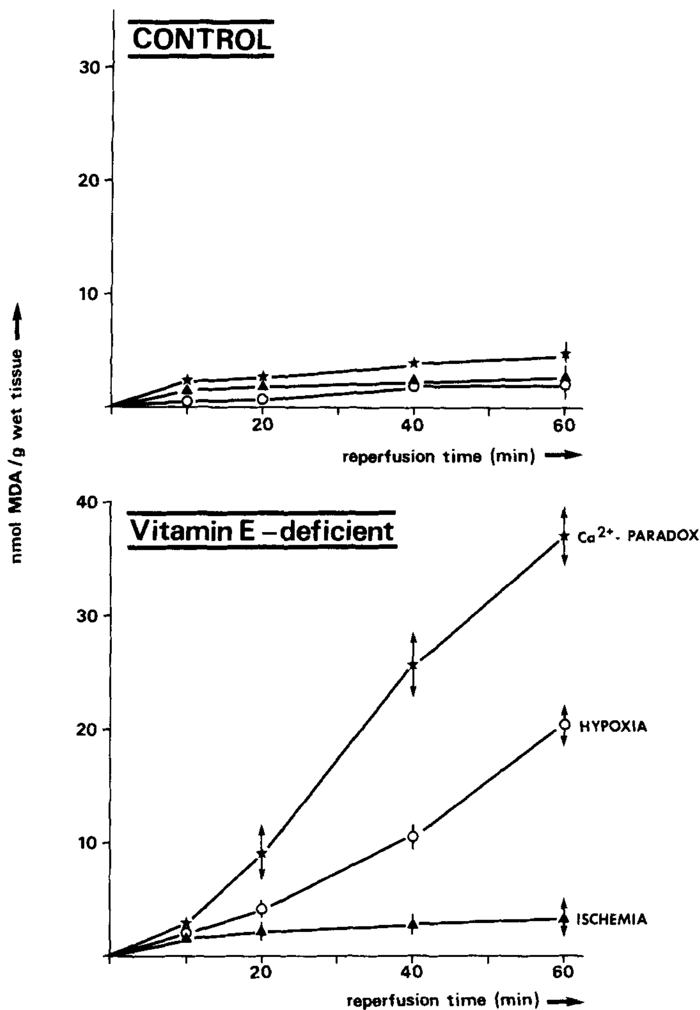

FIGURE 3 Cumulative appearance of TBA-rm, expressed in nmol malondialdehyde (MDA), in the recirculating perfusion fluid during normal reperfusion for 60 minutes after a Ca^{2+}-free (*), hypoxic (o) or ischemic treatment (▲) of control or vitamin E-deficient rat heart. Each value represents the mean ± SEM of at least 4 separate experiments. Significance of difference of vitamin E-deficient versus control heart: $p < 0.001$ both on reperfusion of hypoxic and Ca^{2+}-free treatment.

which leads to an intracellular Ca^{2+}-overload, is a common factor in cardiac necrosis both during reoxygenation after a period of hypoxia or ischemia and the Ca^{2+}-paradox[7,8,27]. We reported before, that the ability to cope with O_2-toxicity is affected when rat hearts are exposed to hypoxic, ischemic or Ca^{2+}-free perfusion and subsequent reperfusion[6]. This observed decrease in protective capacity of myocardial cells and raise in membrane permeability can result from increased oxidative stress as reactive intermediates can inactivate or transform enzymes and cause oxidation of thiols and polymerization of proteins. No peroxidation of lipids was, however,

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detectable even during the Ca^{2+}-paradox, which induced the most prominent loss of protective factors, especially GSH[6]. In the present experiments again no formation of TBA-rm was found after exposing hearts of normally fed rats to the above mentioned treatments. This points at a very effective protection of cardiac membrane lipids.

The formation of TBA-rm both in heart tissue and in the coronary perfusate in vitamin E-deficient hearts during normal reperfusion of hypoxic hearts suggests that this treatment actually increases free radical formation. The difference between ischemia and hypoxia with respect to their ability to enhance free radical formation, is unclear yet. The occurrence of a "no-reflow" phenomenon upon reperfusion has been reported for both treatments[28]. The extent to which the ventricular walls are non-perfused is, however, higher after ischemia. A slower onset of free radical formation with a subsequent lesser attack on the protective mechanisms, as was observed, might result from this when compared to hypoxia. Furthermore it must be kept in mind that a low formation rate can make TBA-rm non-detectable as malondialdehyde is known to bind to proteins and to be metabolized in mitochondria. The absence of an increased formation of TBA-rm therefore not excludes an increased formation of free radicals on reflow after ischemia.

During the Ca^{2+}-paradox phenomenon TBA-rm was detected in vitamin E-deficient rat heart. However, upon this treatment a massive efflux of GSH into the perfusate was detected, which confirms that sarcolemmal permeability is sharply increased following Ca^{2+}-repletion[29,30]. The formation of TBA-rm cannot therefore be unequivocally ascribed to excessive free radical formation directly resulting from a disturbance of the Ca^{2+}-homeostasis, as the normally occurring free radical formation might not be prevented effectively anymore. The peroxidative processes that result may, however, contribute to the irreversible injury of the heart during the Ca^{2+}-paradox phenomenon. The observation that dimethylsulfoxide, a radical scavenger, has a protective action against the Ca^{2+}-paradox injury, points also in this direction[31].

During all above mentioned treatments absence of vitamin E did not impose a higher stress on the cellular protective mechanisms in the heart, since no difference between control and vitamin E-deficient hearts were found in this respect. No higher permeability of the cardiac membranes was found due to the treatment in vitamin E-deficient rat hearts as well. The ratio between the GSH and GSSG content in the perfusate, indicative of increased free radical stress, also remains almost unchanged. In addition, the presence of the lipid-soluble vitamin E can not prevent attack on other defence mechanisms. This implies that lipid peroxidative processes in cardiac membranes probably are secondary to other deleterious effects which occur due to the treatments studied. Supplementation of especially the water-soluble protective factors seems necessary in order to retain the ability to cope with O_2-toxicity. On the other hand degradation of membranes could not be prevented either during the Ca^{2+}-paradox or upon reoxygenation after hypoxia when vitamin E is not present. This points at the importance of the presence of sufficient vitamin E in cardiac membranes. Repair of vitamin E-free radicals is however dependent on water-soluble protective factors like GSH[32] and vitamin C[33,34]. In this way replenishment of water-soluble defence factors would additionally provide protection to membranes. It must further be investigated, e.g. by studying heart perfusion models which induce reversible damage, whether a reduction of function and structural impairment can be found by such replenishment.

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Accepted by Dr. B. Halliwell

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