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DECREASED DEFENCE AGAINST FREE RADICALS IN RAT HEART DURING NORMAL REPERFUSION AFTER HYPOXIC, ISCHEMIC AND CALCIUM-FREE PERFUSION

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Summary

Excessive formation of free radicals possibly plays an important role in the origin of irreversible damage of the heart after hypoxic, ischemic or Ca^{2+}-free treatment. The effect of these treatments on the activity of superoxide dismutase and the glutathione system was studied on isolated rat heart. These activities reflect the protective capacity of the heart against reactive substances. In addition the peroxidation of lipids is determined in the treated hearts using malondialdehyde formation as an indicator. All experiments were performed using a Langendorff-apparatus with recirculating perfusion.
The observed changes in the components of the glutathione system and superoxide dismutase activity both after hypoxic, ischemic and Ca^{2+}-free perfusion, as measured upon reperfusion, indicate a decrease in cellular defense mechanisms in the heart against free radicals. The effect was most pronounced upon Ca^{2+}-repletion after a period of Ca^{2+}-free perfusion. No malondialdehyde could however be detected either in the tissue of the treated hearts or in the perfusate. Our data give reason to expect beneficial effects of an adequate pharmacological treatment, which replenishes the cellular defence systems.

Myocardial cells are damaged in a severe, irreversible way as a result of prolonged ischemia, hypoxia or of a Ca^{2+}-free perfusion, followed by normal reperfusion of the myocardium. Alhtough the nature of these damaging processes differs, there are striking similarities in the way they lead to final cell necrosis (1-3). Reperfusion of the myocard after a period of hypoxia, ischemia or Ca^{2+}-depletion, leads to profound alterations in cell structure and function (4-6), causes a sudden release of cytoplasmic enzymes (7) and disturbs the normal intracellular Ca^{2+}-homeostasis, leading to an intracellular Ca^{2+}-overload (8, 9). In addition, a disregulation of the ATP-production in the mitochondria occurs (10).

Recent literature data indicate that hypoxic, ischemic or Ca^{2+}-free treatment of the heart leads to an increased formation of free radicals in the myocard tissue, mainly reactive O_2-species (11-21). The protection from O_2-toxicity in heart cells is provided by superoxide dismutase (SOD), which catalyzes the dismutation of $O_2^{\cdot-}$ to H_2O_2 in cytoplasma and mitochondria. Because of the low catalase activity in heart cells, the further degradation of H_2O_2 to H_2O is catalyzed mainly by glutathione-peroxidase (GSH-Px), using GSH as a hydrogen donor. This leads to interconversion of GSH into its oxidized form, GSSG.

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In the present study we investigated the effect of hypoxic, ischemic and Ca^{2+}-free perfusion followed by normal reperfusion on the activity of SOD and the glutathione system. As peroxidation of lipids occurs when the protective factors in tissues can not handle an increase in free radical generation, we further investigated whether (one of the) above mentioned treatments actually lead to insufficient protection against oxidative stress in the heart. The formation of malondialdehyde (MDA) was used as a parameter for lipid peroxidation. As cumene hydroperoxide is known to disturb the balance between the protection against free radicals on the one hand and the generation of reactive substances on the other in heart tissue (22), perfusions with cumene hydroperoxide were performed as a reference treatment.

Materials and Methods

Perfusion method: Male Wistar rats (200-250 g) 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 (23). 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 min 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 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. In case of the 0.3 mM cumene hydroperoxide perfusion, cumene hydroperoxide was added to the recirculating standard salt solution. At the indicated sample-times 2 ml of the perfusate were collected. At the end of the perfusion the heart tissue was rapidly frozen using Wollenberger clamps precooled in liquid nitrogen (24).

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 GSH-Px activity was assayed in the homogenate according to the method of Lawrence and Burk (25). 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 (26). According to this method the amount of GSSG was measured after binding the GSH by 2-vinylpyridine. The procedure of Weisiger and Fridovich (27), using the cytochrome c/xanthine oxidase system, served as a method for the determination of SOD. The amount of lipid peroxidation was assayed in heart homogenate and perfusate by the thiobarbituric acid test, according to Buege and Aust (28). 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.

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

Results

Hypoxic, ischemic or Ca^{2+}-free perfusion with subsequent recirculating reperfusion with standard salt solution for 60 minutes, resulted in reduction of SOD activity in rat heart (Fig. 1). The effect was most pronounced upon Ca^{2+}-repletion of the heart, the so-called "Ca^{2+}-paradox experiment". A recirculating perfusion with a 0.3 mM cumene hydroperoxide solution also diminished the $O_2^{\cdot-}$-dismutating capacity of the heart. The GSH-Px axtivity of rat hearts was not affected by ischemic treatment followed by normal reperfusion (Fig. 2). After hypoxic perfusion and subsequent reoxygenation, the GSH-Px activity slightly increased, albeit not significantly ($p < 0.1$). Both exposing the hearts to a Ca^{2+}-paradox and perfusion with cumene hydroperoxide led to a significant decrease in GSH-Px activity.

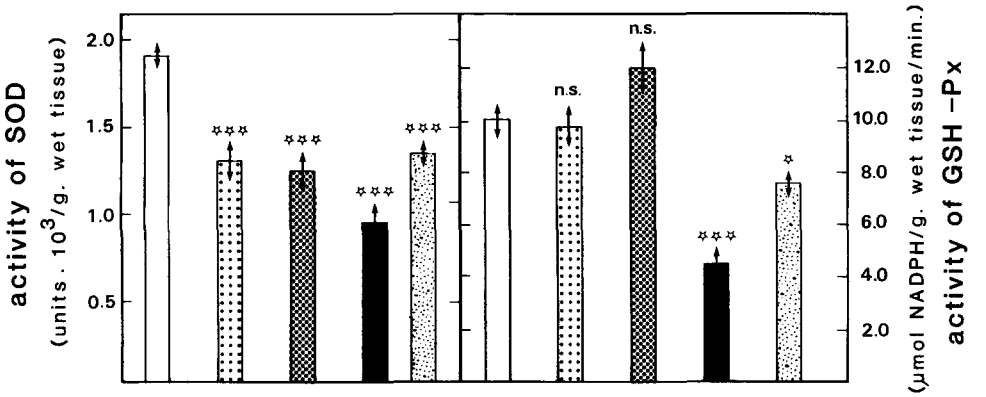

Figure 1 Figure 2

The effect of a recirculating perfusion during 60 minutes on the activity of SOD (Figure 1) and GSH-Px (Figure 2) in the heart. □ control, ⊡ ischemic, ▩ hypoxic, ■ Ca^{2+}-free and ⊡ cumene hydroperoxide treatments. Each result is the mean ± SEM of six separate experiments. Significance of differences from control value: n.s. = not significantly, ★= $p < 0.01$ and ★★★= $p < 0.001$.

Table I shows the cumulative release of GSH and GSSG into the coronary effluent during the recirculating perfusion of rat heart with standard salt solution. The level of GSH and GSSG in the perfusate of control hearts remained low. In contrast, hearts exposed to hypoxia and ischemia released both GSH and GSSG into the effluent upon normal reperfusion. In hearts repleted with Ca^{2+}-ions after Ca^{2+}-free perfusion, the loss of GSH to the effluent was already very high 20 minutes after the start of Ca^{2+}-reperfusion. This GSH content of the perfusate diminished with the duration of the perfusion in contrast with the GSH release during reoxygenation, which increased with time. The GSSG level in the perfusate raised with time both during reperfusion after hypoxia, ischemia and Ca^{2+}-depletion. In case of the 0.3 mM cumene hydroperoxide perfusion the content of GSH and GSSG in the effluent also increased with time.

The total amount of glutathione in heart tissue after the different treatments, as reported in Fig. 3, was significantly reduced in all cases, as compared to control hearts. The effect was most pronounced when hearts were suddenly reconfronted with Ca^{2+}-ions after a Ca^{2+}-free period. In all cases the major part of the total glutathione content in the heart tissue consisted of GSH. The GSSG concentration remained very low.

TABLE I

Treatment followed by normal reperfusion	20 min - *reperfusion time* - 60 min			
	GSH	GSSG	GSH	GSSG
	(expressed as nmol/g wet tissue/total perfusate)			
control	1 ± 0.8	2 ± 0.8	9 ± 0.3	9 ± 3
ischemic	49 ± 22	44 ± 17	112 ± 43	128 ± 43
hypoxic	84 ± 35	56 ± 35	103 ± 56	141 ± 67
Ca^{2+}-free	621 ± 46	308 ± 40	196 ± 39	484 ± 47
CuOOH	40 ± 8	121 ± 6	194 ± 40	312 ± 24

The cumulative release of GSH and GSSG into the perfusate measured *upon normal reperfusion* after an ischemic, hypoxic or Ca^{2+}-free period as compared to control and cumene hydroperoxide (CuOOH) treatments. Each value represents the mean ± SEM of six separate experiments. All values are significantly different from the control.

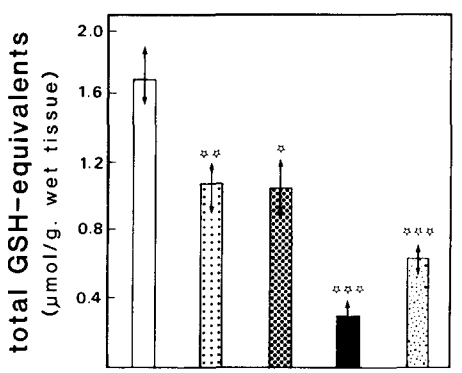

FIGURE 3

The total glutathione content (GSH + GSSG, expressed as GSH-equivalents) in heart tissue after a recirculating perfusion during 60 minutes. □ control, ⊡ ischemic, ▩ hypoxic, ■ Ca^{2+}-free and ⊠ cumene hydroperoxide treatment. Each value represents the mean ± SEM of six separate experiments. Significance of differences from control value: ★= $p < 0.02$, ★★ = $p < 0.002$ and ★★★= $p < 0.001$.

Perfusion with the cumene hydroperoxide solution led to profound lipid peroxidation, as measured by the presence of MDA in the perfusate (Fig. 4). The formation of MDA increased with time and reached a plateau within 60 minutes. In contrast, no thiobarbituric acid reactants could be detected in the perfusate when rat hearts were exposed to a recirculating reperfusion after a hypoxic, ischemic or Ca^{2+}-free period. In heart tissue MDA was found neither during normal reperfusion after hypoxia, ischemia or a Ca^{2+}-free period, nor after a perfusion with cumene hydroperoxide.

Expression of all above mentioned data per mg protein leads to similar results.

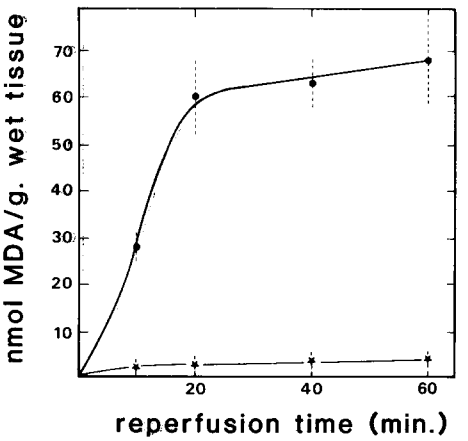

FIGURE 4

Time course of the cumulative release of MDA into the perfusate during recirculating perfusion of rat heart with 0.3 mM cumene hydroperoxide solution (●) and during normal reperfusion following ischemic, hypoxic, Ca^{2+}-free or control perfusion (✱). Each value represents the mean ± SEM of six separate experiments.

Discussion

The possible involvement of radicals in the induction of tissue damage upon reperfusion of hypoxic, ischemic or Ca^{2+}-depleted hearts, is recently under investigation. Several literature data indicate that an explosive formation of active oxygen substances occurs upon normal reperfusion after a period of ischemia or hypoxia (17, 19, 29). In addition, big disturbances in the Ca^{2+}-homeostasis, which are not only seen during the Ca^{2+}-paradox but also during hypoxic and ischemic treatment, are reported to cause an enhancement of free radical production (9, 30, 31). For example, the extra activation of lipid chemiluminescence upon reperfusion after myocardial ischemia (15), similarities between ischemic damages in cardiac cells and those induced by catecholamines, able of forming radicals (11), and the capability of free oxygen radicals to reproduce the changes seen in sarcoplasmic reticulum from ischemic myocardium (13, 14), all suggest that radicals play an important role in the development of cardiac necrosis.

Our findings point at a reduced SOD activity in the tissue both after hypoxic, ischemic and Ca^{2+}-free perfusion followed by reperfusion, the effect being most severe during the Ca^{2+}-paradox experiment. As no SOD activity could be detected in the perfusate, partly inactivation of the enzyme must have occurred. H_2O_2 is known to inactivate SOD when this reactive species can not be eliminated in time (32). As a consequence of a reduced SOD activity very reactive OH·-radicals can be formed by the Fenton reaction (the modified Haber-Weiss reaction):

$$O_2^{-\cdot} + H_2O_2 \xrightarrow{Me^{n+1}} O_2 + OH^{\cdot} + OH^{-}$$

These highly reactive OH·-radicals will strongly enhance the oxidative stress on the heart tissue.

As far as GSH-Px is concerned, our results indicate that the heart had the same (after ischemia) or even an inclination towards a higher capacity (after hypoxia) to handle H_2O_2 or lipid hydroperoxides. Elevation of the GSH-Px activity is commonly associated with small increments in oxidative stress (33-35), an aspecific response to injury, however, cannot be excluded. During the Ca^{2+}-paradox phenomenon the GSH-Px activity is strongly reduced; no activity of GSH-Px could be detected in the perfusate.

The total amount of glutathione in the heart tissue was prominently lowered after hypoxic, ischemic and Ca^{2+}-free treatment, which may have led to a shortage of co-factor supply in the glutathione system.

Release of GSH and GSSG into the perfusate was seen upon all treatments. The release of GSH into the perfusate indicates an increased permeability of cell membranes. During the Ca^{2+}-paradox experiment a massive efflux of GSH occurred shortly after the start of the normal reperfusion which reaffirms the strong increase of sarcolemmal permeability after Ca^{2+}-repletion, previously observed by Zimmerman and Hülsmann (36) and Koomen et al. (37). The subsequent decrease of the GSH-content in the perfusate with time is an indication for its conversion into GSSG during the recirculating perfusion. Also the GSSG-release upon reoxygenation might, in part, result from such a conversion, as other biochemical data on the reoxygenation phenomenon only revealed a significant increase in GSH-release and not in GSSG-release (12). On the other hand GSSG-release from the cells would not be surprising since it might also reflect an increased intracellular concentration of GSSG due to an enhanced flux through GSH-Px, as measured upon reoxygenation. Exposure to oxidative stress generally leads to increased formation of GSSG (38), the rate of GSSG-release being indicative of its intracellular concentration (39).

Considering the important role of glutathione in many biological processes in the cell, changes in the GSH/GSSG ratio may give early indications of deterioration of myocardial function. Moreover, the observed changes in SOD and GSH-Px activity in combination with the alterations in GSH/GSSG concentration suggest that insufficient protection against reactive substances worsens in time, especially during the Ca^{2+}-paradox. Peroxidative degradation of lipids is however not substantiated by MDA formation in our experiments. Yet, it must be kept in mind that metabolism in mitochondria or binding to proteins (40) can make MDA non-detectable in whole organs. Other investigators demonstrated that reoxygenation after a period of hypoxia actually enhances MDA formation in rat heart (12, 19). Presence of glucose in the standard salt solution is known to retard the induction of damage due to hypoxic perfusion. A lowering of the formation rate of free radicals due the presence of glucose might explain the absence of detectable MDA in our reoxygenation studies. Possibly MDA is only a sensitive indicator for lipid peroxidative processes in the isolated, perfused rat heart, when the formation rate of radicals is high, as seen during cumene hydroperoxide perfusion.

In conclusion, our results indicate 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. The findings provide a rationale for the recently reported improvement or even prevention of functional and morphological deterioration, due to hypoxia, ischemia and/or Ca^{2+}-depletion by reinforcements of the protective mechanisms against free radicals in the heart, e.g. Na selenite (16), CoQ (41), DMSO (42) and HP-6, an antioxidant belonging to the hydroxypyridine series (18). Further beneficial effects can be expected from application of sulfhydryl donors like N-acetylcysteine.

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