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CIRCADIAN VARIATIONS IN ANTIOXIDANT DEFENCES AND LIPID PEROXIDATION IN THE RAT HEART

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Circadian variations in antioxidant defences and lipid peroxidation were investigated in 12 rat hearts perfused during light (i.e., at 08.00, n = 6) and dark cycle (i.e., at 19.00, n = 6). Higher levels of non proteic thiol compounds ($P < 0.01$), glutathione transferase activity ($P < 0.05$) and lipid peroxidation ($P < 0.01$) were detected in evening-excised hearts, associated with a lower ($P < 0.05$) selenium-dependent glutathione peroxidase activity; superoxide dismutase and glutathione reductase activities, as well as vitamin E content, were similar in the two groups. Moreover, a greater release of thiobarbituric acid reactive substances ($P < 0.01$) and proteins ($P < 0.05$) was detected in the myocardial effluent of another group of 5 evening-excised hearts perfused with Krebs-Henseleit buffer containing 30 μM cumene hydroperoxide, as compared to 5 light-cycle hearts.

In conclusion, a higher oxidative stress seems to be operative in the rat heart during early stages of the dark phase, in spite of the increased level of non proteic thiol compounds (namely, glutathione). An imbalance of antioxidant defences, and/or higher radical generation and unsaturation degree of biomembranes lipids, may be hypothesized to favour myocardial oxidative stress at the beginning of the motor activity phase in rats.

KEY WORDS: Circadian, free radicals, antioxidants, glutathione, lipid peroxidation, rat heart.

INTRODUCTION

It is known that mammalians are subjected to circadian fluctuations of many functional and biochemical processes, to allow proper adaptive responses throughout the day.[1]

Experimental evidences suggest that also cell oxidant burden may show circadian variations. Accordingly, increased mitochondrial oxygen radical generation has been reported during the dark cycle in the rat,[2] an animal species with a nocturnal motor activity phase. Indeed, the hypermetabolic status of the activity phase may favour enhanced radical generation especially in the heart and muscle, in light of the relationship between the degree of tissue metabolism and radical production at subcellular level.[3,4] However, several lines of antioxidant defences are devoted to protect the cell against free radical attack, which promotes lipid peroxidation and

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altered biomembranes permeability.[5] Even though GSH levels have been shown to fluctuate during the day in the rat heart,[6] little is known concerning circadian variations of the wide spectrum of enzymatic and non-enzymatic antioxidant defences and of the lipid peroxidation in the mammalian myocardium. In the present paper, this issue was specifically addressed in perfused rat hearts, which were also subjected to peroxide challenge during the light and dark cycle, to establish potential relationships between prooxidant/antioxidant status and functional response to oxidative stress of the myocardium during the day.

MATERIALS AND METHODS

Reagents, animals and heart perfusion

Reagents were purchased from Sigma Chemical Co., St. Louis, MO.

Twelve male, Sprague Dawley rats (320–350 g) were housed in cages containing no more than three animals and acclimated for 2 weeks under a normal lighting schedule (L: 06.00–18.00; room temperature 26°C). After the acclimation period, only one rat in the light cycle (at 08.00) and one in the dark cycle (at 19.00) were daily sacrificed by decapitation after diethylether anesthesia. The study was conducted in the early stages of rat activity phase (i.e., at 19.00) since there is clinical evidence of higher susceptibility to potentially radical-related cardiovascular fatalities, such as myocardial infarction and sudden cardiac death, soon after awakening.[7,8] The hearts were retrogradely perfused on a double reservoir, gravity-flow Langerdorff apparatus operating at 75 mm Hg and kept at 37°C. The perfusion medium was a bicarbonate Krebs-Henseleit buffer (KHB), containing 11 mM glucose and gassed with 95% O_2 and 5% CO_2 (pH 7.4). Resting tension (RT) and developed tension (DT) were recorded by a force transducer (model 7004, Basile, Italy) tied to the apex of the left ventricle and connected to a multichannel Sensormedics Dynograph R 611 recorder. To allow a proper hemodynamic study, heart rate was kept constant at 240 beats/min by right pacing. Coronary flow (CF) was measured by one min perfusate collections. The hearts used for tissue biochemical studies in basal conditions were perfused with oxygenated KHB for 30 min, to completely remove the blood from the myocardium.

For specific studies regarding the evaluation of circadian heart susceptibility to oxidative stress, another two groups of 10 rats were sacrificed at 08.00 (n = 5) and 19.00 (n = 5), and the hearts, after a 30 min "stabilization" period, were perfused for 40 min with KHB containing 30 μM cumene hydroperoxide (CHP), recording hemodynamic parameters and collecting the myocardial effluent.

Thiobarbituric acid reactive substances (TBARS) and protein release in the myocardial effluent of hearts perfused with CHP

TBARS were assessed with acidified thiobarbituric acid (TBA), (15% w:v trichloroacetic acid, 0.375% w:v TBA, 0.025 N HCl), as previously reported.[9] After boiling for 15 min and cooling, the chromogen was extracted with n-butanol, followed by centrifugation at 1,000 × g for 10 min, and read at 532 nm. Values were expressed as nmol TBARS/40 min/g wet tissue, using a molar extinction coefficient of $1.54 \times 10^5\ M^{-1}\ cm^{-1}$.[5]

Protein perfusate release, as well as tissue protein concentrations, were assessed by the Bradford's method,[10] expressing the release as mg/40 min/100 mg tissue protein.

Tissue biochemical analyses

The ventricular myocardium was homogenized (1:6 w/v) in ice-cold 0.05 M potassium phosphate buffer (pH 7.4), containing 1 mM EDTA, followed by a first centrifugation at 800 × g for 3 min at 4°C to assess vitamin E, non proteic thiol compounds (NP-SH) and lipid peroxidation, and a second centrifugation at 2,000 × g for 10 min to assay superoxide dismutase (SOD; EC 1.15.1.1.) activity. Glutathione reductase (GR; EC 1.6.4.2.), Glutathione peroxidase (GSH-Px; EC 1.11.1.9) and Glutathione transferase (GST; EC 2.5.1.18.) activities were measured on cytosol harvested after further centrifugation at 105,000 × g for 60 min.

The total SOD activity was measured as previously described,[11] using 0.2 mM pyrogallol in Tris-HCl buffer, pH 8.2, containing 0.8 mM EDTA. Specific activity was expressed as Units (U)/mg protein, each U representing the amount of enzyme causing a 50% inhibition of pyragallol autooxidation at 25°C.[11]

NP-SH (of which GSH represents virtually the only pool in the heart[12]) were determined after extraction with 4% sulfosalicylic acid and specific reaction with 0.1 ml of 15 mM 5,5'-dithiobis-2-nitrobenzoic acid, as previously described.[13] After recording absorbance values at 412 nm with a double beam Varian DMS 200 spectrophotometer, NP-SH values were calculated as nmol NP-SH/g wet tissue (molar extinction coefficient: $13.6 \times 10^3\ M^{-1}\ cm^{-1}$).

For GR activity measurement, an appropriate amount of cytosol (0.05–0.1 ml) was added to the assay mixture (1.0 ml) containing 0.1 M potassium phosphate buffer, pH 7.4, 1 mM GS-SG, 1 mM EDTA, and 0.16 mM NADPH.[13] The NADPH disappearance was followed at 340 nm and 37°C. One activity unit (U) represents 1 μmole of NADPH oxidized/min (NADPH extinction coefficient: $6.22 \times 10^3\ M^{-1}\ cm^{-1}$).

GSH-Px activity was assayed essentially by the Paglia and Valentine's procedure.[13,14] The assay solution (2.0 ml) contained 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA and 1.5 mM NaN_3, 1 mM GSH, 0.16 mM NADPH, 4 μg of glutathione reductase, a suitable cytosol amount and 0.25 mM H_2O_2 as substrate. The reaction rate was recorded at 37°C, following the decrease in absorbance of NADPH at 340 nm. One activity unit (U) is 1 μmole NADPH oxidized/min.

GST activity was determined by following at 340 nm the rate of conjugation of GSH with 1-chloro-2,4 dinitrobenzene (CDNB), according to the Habig's method,[15] with slight modifications as previously reported.[13] One activity unit (U) is the amount of the enzyme conjugating 1 μmole of substrate/min/mg protein.

The glutathione peroxidase activity of GST (namely, selenium-independent glutathione peroxidase: GST-Px) was investigated by Lawrence and Burk's method,[16] using 1.2 mM CHP as substrate in the same assay system of GSH-Px, but replacing CHP for H_2O_2.[13]

Vitamin E content was assessed by the method of Taylor *et al.*,[17] after homogenates saponification for 30 min at 70°C with 1.0 ml of 10 N KOH, 1.0 ml of absolute ethanol and 0.5 ml of 0.25% ascorbic acid, followed by hexane extraction. The organic phase was fluorimetrically measured at 286 excitation and 330 emission with a Perkin Elmer LS-5 spectrofluorimeter, calibrated with 1 μg of quinine sulfate/ml of 0.1 N H_2SO_4.[17]

Values were expressed as μg α-tocopherol (used as the external standard)/g wet tissue.

Lipid peroxidation was also investigated by TBARS assessment. However, another TBA-independent method (i.e., study of fluorescent damage products of lipid peroxidation) was used to maximize peroxidation study specificity.[5] TBARS were measured according to the modified method of Ohkawa *et al.*[18] An aliquot of the homogenate was added to 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid solution (pH 3.5), 1.5 ml aqueous solution of 0.8% TBA and 1 μmole of butylated hydroxytoluene in ethanol as antioxidant. After heating at 95°C for 60 min and cooling, the chromogen was extracted with n-butanol plus pyridine (15:1, v/v) and, after a brief centrifugation, read at 532 nm against an appropriate blank. Values were expressed as nmol TBARS/g wet tissue, using for calculations a molar extinction coefficient of $1.54 \times 10^5\ M^{-1}\ cm^{-1}$.[5]

Fluorescent damage products of lipid peroxidation (FDPL), a sensitive *in vivo* index of oxidative stress,[12] were assessed as previously reported,[12,19] after homogenate lipid extraction with chloroform/methanol (2:1, v/v). "Aqueous" and "chloroform" phases were separated by the addition of bidistilled water to the chloroform/methanol extract and centrifugation for 5 min. The lipid-containing phase was subjected to fluorimetric measurement at 355 nm excitation and 450 nm emission with a Perkin Elmer LS-5 spectrofluorimeter (excitation and emission slit widths at 10 nm; temperature 25°C). Values were expressed as units of relative fluorescence (URF)/g wet tissue.

Statistical analysis

Results were calculated as means ± SD. Unpaired Student's t test was used to compare hemodynamic and biochemical data between the two groups. $P < 0.05$ was regarded as statistically significant.

RESULTS

Tissue biochemical parameters

Tissue biochemical parameters of morning- and evening-excised hearts are summarized in Table I. GST activity ($P < 0.05$), as well as NP-SH ($P < 0.01$) and both TBARS and FDLP ($P < 0.01$), were significantly higher, whereas GSH-Px activity was lower ($P < 0.05$), in the early stages of dark cycle. Vitamin E content and the activity of total SOD and GR were similar in the two groups, which also show no detectable selenium-independent glutathione peroxidase activity.

Heart hemodynamics and effects of CHP perfusion

After 30 min of aerobic perfusion, CF and DT values were 5.9 ± 1.2 vs 6.25 ± 1.45 ml/min/g wet tissue, and 3.5 ± 0.6 vs 3.7 ± 0.5 gr in morning- and evening-perfused hearts, respectively (P = NS), whereas RT remained at "zero" level in the two groups.

During CHP perfusion, both groups underwent an irreversible contracture, with virtually undetectable DT values, after about 12 min (P = NS), CF drop being similar (i.e., about −60%, P = NS) in the two groups at the end of peroxide perfusion. In spite of this lack of differences in heart hemodynamics, higher values of TBARS

TABLE I
Circadian variations in antioxidant defences and lipid peroxidation of the rat heart

	Morning (08.00)	Evening (19.00)
SOD	3.9 ± 0.2	4.05 ± 0.4
NP-SH	283 ± 54	429 ± 65**
GR	0.020 ± 0.001	0.021 ± 0.0015
GSH-Px	0.590 ± 0.03	0.555 ± 0.02*
GST	0.105 ± 0.01	0.129 ± 0.02*
GST-Px	ND	ND
Vit E	4.5 ± 1	4.8 ± 0.8
TBARS	15.5 ± 2.5	21 ± 3**
FDPL	122 ± 27.5	177.5 ± 24**

SOD: Total superoxide dismutase activity (U/mg protein); NP-SH: Non proteic thiol compounds (nmol NP-SH/g wet tissue); GR: Glutathione reductase activity (U/mg protein); GSH-Px: Selenium-dependent glutathione peroxidase activity (U/mg protein); GST: Glutathione transferase activity (U/mg protein); GST-Px: Selenium-independent glutathione peroxidase activity (U/mg protein); Vit E: Vitamin E content (μg α-tocopherol/g wet tissue); TBARS: Thiobarbituric acid reactive substances (nmol TBARS/g wet tissue); FDPL: Fluorescent damage products of lipid peroxidation (units of relative fluorescence, URF/g wet tissue). Values express the mean ± SD of 6 hearts for each group.

* $P < 0.05$, and ** $P < 0.01$ vs morning-excised hearts; ND: not detectable.

(158 ± 17 vs 129 ± 14 nmol TBARS/40 min/g wet tissue, $P < 0.01$) and proteins (1.6 ± 0.4 vs 1.1 ± 0.25 mg protein/100 mg tissue protein, $P < 0.05$) were detected in the myocardial effluent of evening-perfused hearts.

DISCUSSION

The present study demonstrates that antioxidant defences and lipid peroxidation of the rat heart show significant circadian variations, with higher susceptibility to oxidative stress in the early stages of dark motor activity phase.

Increased evening levels of NP-SH (namely, GSH) agree with previous reports,[6] though causal mechanisms remain not yet fully explained. However, enhanced GSH generation from GS-SG is not involved, since GR activity does not differ in the morning- and evening-excised hearts. Thus, increased uptake or biosynthesis, and/or reduced degradation of GSH should be operative. In this regard, it is known that plasma catecholamines and vasopressin rise during the afternoon in the rat.[20,21] Vasopressin and catecholamines may induce liver GSH release,[22] thus favouring muscle tissue GSH uptake.[23] This "interorgan" mechanism could provide antioxidant supply to working hypermetabolic myocytes, when more radicals are generated at subcellular level. In this context, depressed GSH liver content,[2] and increased heart rate[24] and metabolic levels[25] have been reported during the dark cycle in rodents.

It could seem a paradox that hearts with higher GSH content are apparently more susceptible to oxidative stress, as judged by enhanced tissue lipid peroxidation, and

greater TBARS and protein release during peroxide perfusion. However, the rise of GSH could not be necessarily adequate to the dark cycle free radical generation of the heart, which may be supported by higher plasma levels[20] and myocardial turnover of catecholamines[26] (well known free radical generating molecules[5]), microsomal hyperfunction,[2] and enhanced tissue metabolic activity,[3,4] with higher oxygen flux and radical generation especially in mitochondria.[2] On the other hand, even though GSH has been emphasized as an endogenous tissue antioxidant,[5] experimental reports suggest that it may also act as a prooxidant via direct interaction with cellular iron.[27] Moreover, it should be noted that the balanced function of antioxidant defences is the key factor in providing cell protection against free radical attacks. In this regard, if GSH rise is not accompanied by a concomitant increase in SOD activity[28] and/or vitamin E content,[29,30] the antilipoperoxidative potential of GSH could also be ineffective. Accordingly, no differences in SOD activity and vitamin E levels are detectable in morning- and evening-excised hearts, whereas increased levels of both TBARS and FDPL are assessed in dark cycle-perfused hearts. The latter aspect agrees with the findings of Diaz-Munoz *et al.*, demonstrating increased lipoperoxidation levels in the rat cerebral cortex during the dark phase.[31] The slight but significant depression of GSH-Px (described also in the rat cerebrum during evening[31]) can also favour higher lipid peroxidation levels in dark-cycle perfused hearts, in light of the recognized antioxidant role of GSH-Px.[32] Another mechanism potentially involved in the increased tissue peroxides content could be related to higher evening levels of corticosteroids,[1,21] which can inhibit cell phospholipase A_2,[33] an enzyme that allows membrane lipid hydroperoxide release for GSH-Px detoxication.[34] Regarding the hyperactivity of GST (which has been reported also in the mice liver during the dark phase),[35] glutathione-S-conjugated (byproducts of GST activity) may exert negative metabolic effects on the heart, such as a specific impairment of GSSG excretion,[36] so potentially resulting in enhanced cell oxidant injury.

In conclusion, rat hearts perfused during the early stages of motor activity phase are apparently more susceptible to oxidative stress, in spite of their higher GSH levels. Further studies are advisable to investigate other mechanisms potentially involved in circadian variations of myocardial vulnerability to oxidant damage, such as tissue radical generation and oxidizable compound pool (i.e., polyunsaturated fatty acids); in this regard, increased radical production and higher lipid unsaturation degree have been reported in the rat liver and brain during the dark cycle.[2,31] A large body of evidence has stressed the role of free radical-mediated injury in the pathophysiology of ischemic heart diseases.[5] Even though caution is needed before extrapolating our results to clinical conditions, the present study could add insights into the open problem of the greater susceptibility to acute cardiovascular fatalities, such as myocardial infarction and sudden cardiac death,[7,8] during early motor activity phase of humans.

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