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Transmural distribution of iron in the hypoxic and reoxygenated rabbit left ventricular myocardium

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Abstract

Transmural distribution of low molecular weight iron (LMWI), total iron, and protein carbonyls (PC) was investigated in the perfused rabbit heart under aerobic conditions, and after 60 min hypoxia followed or not by 3 min reoxygenation. In the aerobic perfused hearts, LMWI, total iron and PC did not show significant transmural differences. Hypoxia increased LMWI and PC levels, which were significantly higher in the subendocardium than in the subepicardium; further significant changes were not observed after reoxygenation. Total iron showed no transmural difference and was not significantly affected by both hypoxia and reoxygenation. Free iron was undetectable in the myocardial effluent of all experimental groups. Thus, hypoxia favors myocardial iron decompartmentalisation and oxidative stress, which are significantly greater in the inner than in the outer ventricular layers. Such findings may add further insight into the problem of the vulnerability of the mammalian subendocardium to injury induced by oxygen deprivation.

Introduction

It is known that injury is greater in the subendocardium than in the subepicardium after ischemia-reperfusion (Reimer *et al.*, 1983; Braunwald & Kloner, 1985), suggesting that the inner ventricular layers are more vulnerable to oxidative stress. In this regard, we have shown that the rabbit heart subendocardium is endowed with a lower antioxidant capacity than the subepicardium, and it is more susceptible to oxidative damage (Lapenna *et al.*, 1994). Moreover, free radical generation is higher in the subendocardium than in the subepicardium of the ischemic-reperfused canine myocardium (Hoshida *et al.*, 1988). Since oxidant generation is fostered by catalytic transition metals, such as iron (Halliwell & Gutteridge 1990), a differential distribution of the metal across the left ventricular wall may be hypothesized. Under physiological conditions, only a minute quantity of the intracellular iron is 'free', namely present as low molecular weight iron (LMWI) form, which is able to induce oxidant damage (Halliwell & Gutteridge 1990; Healing *et al.*, 1990; Voogd *et al.*, 1992). Although LMWI has been reported to increase in the ischemic rabbit kidney (Healing *et al.*, 1990) and in the ischemic rat heart (Voogd *et al.*, 1992), no data are currently available about its transmural distribution in the mammalian myocardium under physiological and, especially, pathological conditions, such as oxygen deprivation and readmission. Investigation into this issue is hampered *in vivo* by the presence of blood and extramyocardial iron in the heart; this problem may be however overcome using a specific experimental model such as the perfused heart. In the present paper we have therefore studied transmural distribution of iron in perfused rabbit hearts under aerobic condi-

tions and after hypoxia with or without reoxygenation; biomolecular oxidant damage was also assessed across the left ventricular wall.

Materials and methods

Experimental protocol

Reagents were from Sigma-Aldrich s.r.l., Milano, Italy. Glassware was steeped overnight in 5 M HCl and then repeatedly rinsed in glass-bidistilled water. Solutions were prepared using Chelex 100 resin and deionized, glass-bidistilled water.

New Zealand rabbits weighing about 2.3 kg were used in the study. Six control hearts were perfused aerobically for 93 min on a double reservoir, Langendorff apparatus operating at 80mmHg and kept at 37 °C (Lapenna *et al.*, 1993; Lapenna *et al.*, 1994). The perfusion medium was a Krebs-Henseleit buffer composed by (in mM) 120 NaCl, 4.8 KCl, 1.0 KH_2PO_4, 2.4 $CaCl_2$, 1.2 $MgSO_4$, 25 $NaHCO_3$ and 5.5 glucose, pH 7.4, and gassed with 95% O_2-5% CO_2, or 95% N_2-5% CO_2, for, respectively, aerobic or hypoxic perfusion (Lapenna *et al.*, 1993). Heart rate was kept constant at 180 beats/min by right ventricular pacing, and myocardial haemodynamic function was monitored measuring resting tension and developed tension as previously reported (Lapenna *et al.*, 1993).

Another 6 hearts were subjected to 60 min hypoxic perfusion, delivered by the second Langendorff reservoir (Lapenna *et al.*, 1993), after 33 min perfusion with normally oxygenated buffer.

A third group of 6 hearts was subjected to 60 min hypoxia followed by 3 min reoxygenation, after 30 min perfusion with normally oxygenated buffer. This reoxygenation time was selected considering that in perfused hearts oxidant generation is maximal in the early stages of oxygen readmission (Zweyer *et al.*, 1989).

Biochemical analyses

Endo- and epi-parts were obtained dividing the free left ventricular wall into four blocks, each of which was in turn cut into subendocardial and subepicardial halves (Lapenna *et al.*, 1994). Specimens were homogenized (1:8, w/v) in 20 mM Tris-HCl buffer, pH 7.6. A first centrifugation at $600 \times g$ was performed to assess total iron and protein carbonyls. A further centrifugation at $105\,000 \times g$ was performed, and the resulting supernatant subjected to ultrafiltration through Amicon 10 000-M_r cutoff filters for LMWI assay; such filters can exclude cytosolic proteins, especially ferritin, from which some iron may be mobilized under acidic analytical conditions.

LMWI was assessed using ferene S, which is the most sensitive iron colorimetric detector (Artiss *et al.*, 1981). Ultrafiltered sample aliquots (0.9 ml) were pretreated with 0.25 ml of 20% trichloroacetic acid (van Jaarsveld *et al.*, 1991); after 10 min, centrifugation at $1,500 \times g$ was performed, and, to 0.9 ml of the supernatant, 0.1 ml of a saturated ammonium acetate solution, 2 mM thiourea, 32 mM ascorbic acid and 0.4 mM ferene S were added. Absorbance values at 594 of the ferene S-iron(II) complex were then recorded spectrophotometrically against an appropriate blank, and results calculated as nmol iron/mg protein using a molar extinction coefficient of 35 500 (Artiss *et al.*, 1981). The detection limit (signal-to-noise ratio = 3) of free iron by such ferene S assay was 0.17 μM, corresponding to about 0.04 nmol iron/mg protein when normalized for the mean sample protein content.

Total iron was measured spectrophotometrically at 535 nm by the bathophenanthroline-isoamyl alcohol method reported by Doeg & Ziegler (1962) and Brumby & Massey (1967), after homogenate treatment with glacial acetic acid plus 5% mercaptoacetic acid and subsequent boiling for 60 min. Results were calculated as nmol iron/mg protein, using a molar extinction coefficient of 19 600 at 535 nm.

Free iron in the myocardial effluent was measured adding to 0.7 ml of the perfusate 0.1 ml of 2 M sodium acetate buffer, pH 4.5, 40 mM ascorbic acid and 0.5 mM ferene S.

Protein carbonyls were determined spectrophotometrically at 370 nm using 2,4-dinitrophenylhydrazine as previously reported (Oliver *et al.*, 1987; Wolfgang *et al.*, 1991). Results were calculated as nmol protein carbonyls/mg protein, using a molar extinction coefficient of 21 000 at 370 nm (Oliver *et al.*, 1987; Wolfgang *et al.*, 1991).

Protein concentrations were assayed by the method of Bradford (1976).

Statistics

Data were calculated as means±SD. Transmural analyte differences were computed by paired Student's *t* test (Glantz 1987; Lapenna *et al.*, 1994), while biochemical differences among aerobic perfused, hypoxic and reoxygenated hearts were analyzed by one-

way analysis of variance (ANOVA) plus Bonferroni's test (Glantz, 1987). A P value less than 0.05 was considered as statistically significant (Glantz, 1987).

Results

Haemodynamic data

In the hearts subjected to hypoxia alone, developed tension was 4.25 ± 0.2 g at baseline, but rapidly declined upon hypoxic perfusion until zero after 60 min hypoxia; at this time, resting tension markedly increased to 7.4 ± 0.5 g. In the hearts subjected to hypoxia and reoxygenation, developed tension was 4.1 ± 0.3 g at baseline, with undetectable values both after 60 min hypoxia and 3 min reoxygenation, which resulted in resting tension values of 7.65 ± 0.6 g.

Biochemical data

As shown in Table 1, there was no significant difference in transmural distribution of LMWI in the aerobic perfused control hearts. Hypoxia increased significantly myocardial LMWI levels, which were higher in the inner than in the outer ventricular layers (Table 1); further significant changes were not observed after reoxygenation (Table 1). Myocardial total iron content did not differ significantly among aerobic control, hypoxic and reoxygenated hearts. Indeed, in these two latter groups the means fell within 12% of the mean values of the control subepicardium (5.15 ± 0.75 nmol/mg protein) and subendocardium (5.2 ± 0.85 nmol/mg protein). Considering these total iron levels, it can be calculated that myocardial LMWI represents about 5% of the total iron pool, which is in agreement with the range of 3–8% previously reported in various rodent tissues (Mulligan *et al.*, 1986).

Protein carbonyls tended to be higher in the subendocardium than in the subepicardium in the aerobic perfused control hearts, although the level of statistical significance was not reached ($P = 0.065$). As compared to controls, protein carbonyl content was approximately doubled in the hypoxic hearts, with higher values in the inner than in the outer layers (Table 1); as for LMWI, further significant changes were not detected after reoxygenation (Table 1).

Finally, free iron was undetectable in the myocardial effluent of all experimental groups.

Discussion

The present study shows that hypoxia induces an increase of myocardial LMWI and protein carbonyl content, which is significantly higher in the subendocardium than in the subepicardium. Hypoxia is known to favor mitochondrial generation of superoxide (Nohl *et al.*, 1993) and tissue accumulation of reducing equivalents (Reimer *et al.*, 1983), which may promote iron delocalization from ferritin (Halliwell & Gutteridge 1990; Reif, 1992). Indeed, iron is bound in the ferric state to ferritin, from which is released as iron(II) by reducing species (Halliwell & Gutteridge, 1990; Reif, 1992). In this regard, it is remarkable that the content of reductants, such as NADH, potentially capable of mobilizing ferritin iron is higher in the subendocardium than in the subepicardium under hypoxic conditions (Minamidate *et al.*, 1973). Moreover, pH is lower in the inner than in the outer ventricular layers during myocardial oxygen deprivation (Watson *et al.*, 1984), conceivably reflecting impaired subendocardial perfusion with lactate accumulation (Humphrey & Gavin 1984; Reimer *et al.*, 1983). Hence, ferritin iron release, which is higher at acidic pH values (Funk *et al.*, 1985), should be favored in the subendocardial myocytes during hypoxia. In such a context, it is of note that, similar to a previous study performed in the ischemic and reperfused rabbit kidney (Healing *et al.*, 1990), tissue total iron content is not significantly affected by both hypoxia and reoxygenation and no iron is lost to the perfusate, suggesting that intracellular iron malplacement leading to LMWI augmentation occurs in the hypoxic myocardium.

Our results also show that 3 min reoxygenation does not change significantly myocardial LMWI and protein carbonyl levels with respect to hypoxia, which appears therefore responsible for myocardial iron decompartmentalisation and oxidative stress of the hypoxic and reoxygenated rabbit heart. These data emphasize the relevance of the hypoxic phase in myocardial oxidant injury associated with hypoxia-reoxygenation, as already pointed out by Park *et al.* (1991) in the perfused rat heart. The hypoxia-dependent impairment of myocardial antioxidant defences (Park *et al.*, 1991; Dhaliwal *et al.*, 1991) may contribute to oxidative stress especially in the subendocardium, which is indeed endowed with a lower antioxidant capacity than the subepicardium (Lapenna *et al.*, 1994). Notably, hypoxic oxidative stress seems to occur mainly in the subendocardium also in the human heart; in fact, it has recently been shown that

Table 1. Low molecular weight iron and protein carbonyls in the control, hypoxic and reoxygenated rabbit hearts.

	Control		Hypoxia		Reoxygenation	
	Endo	Epi	Endo	Epi	Endo	Epi
LMWI	0.27± 0.04	0.26± 0.03	0.57±** 0.07	0.42±*,** 0.05	0.64±** 0.08	0.46±*,** 0.06
PC	4.3± 0.65	4.05± 0.6	8.4±** 0.9	7.3±*,** 0.8	8.1±** 0.77	7.0±*,** 0.7

Endo: subendocardium. Epi: subepicardium. LMWI: low molecular weight iron (nmol/mg protein). PC: protein carbonyls (nmol/mg protein).
Means ± SD of 6 perfused rabbit hearts for each experimental group.
*, $P < 0.05$ vs endo (paired Student's *t*-test).
**, $P < 0.05$ vs aerobic perfused control hearts (ANOVA plus Bonferroni's test).

the content of heme oxygenase-1 (a hypoxia- and oxidative stress-inducible protein) and its decrement by mechanical circulatory assistance are maximal in the innermost ventricular layers of patients with end-stage heart failure (Grabellus *et al.*, 2002). Whether overexpression of heme oxygenase-1, which converts heme into free iron (Suttner & Dennery, 1999), favors subendocardial LMWI augmentation *in vivo* deserves further investigation.

In conclusion, hypoxia results in uneven iron decompartmentalisation and oxidative stress across the rabbit left ventricular myocardium, with occurrence of LMWI and protein carbonyl burden in the innermost layers. These findings could add further insight into the problem of the vulnerability of the subendocardium to injury induced by oxygen deprivation.

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