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Proposed mechanisms for red palm oil induced cardioprotection in a model of hyperlipidaemia in the rat

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Abstract

High-cholesterol diets alter myocardial and vascular NO-cGMP signaling and have been implicated in ischaemic/reperfusion injury. We investigated the effects of dietary red palm oil (RPO) containing fatty acids, carotonoids, tocopherols and tocotrienols on myocardial ischaemic tolerance and NO-cGMP pathway function in the rat. Wistar rats were fed a standard rat chow ± RPO, or a standard rat chow + cholesterol ± RPO diet. Myocardial mechanical function and NO-cGMP signaling pathway intermediates were determined before, during and after 25 min ischaemia. RPO-supplementation improved aortic output recovery and increased myocardial ischaemic cGMP concentrations. Simulated ischaemia (hypoxia) increased cardiomyocyte nitric oxide levels in the two RPO supplemented groups, but not in control non-supplemented groups. RPO supplementation also increased hypoxic nitric oxide levels in the control diet fed, but not the cholesterol fed rats. These data suggest that dietary RPO may improve myocardial ischaemic tolerance by increasing bioavailability of NO and improving NO-cGMP signaling in the heart.

1. Introduction

Nitric oxide (NO) is, by virtue of its vasodilator, antioxidant, anti-platelet and anti-neutrophil actions an essential molecule for normal heart function [1] and has been shown to be cardioprotective in the ischaemic heart [2]. Protective effects of NO are mediated through the production of cGMP. Du Toit et al. [3] found that NO donor treatment reduces ischaemia/reperfusion injury by increasing cGMP concentrations. However, NO has also been shown to be detrimental during pathological conditions such as ischaemia and reperfusion when it reacts with superoxide (O_2^-) to form peroxynitrite ($ONOO^-$) which rapidly decomposes to highly reactive oxidant species leading to tissue injury [1]. Oxygen-free radicals formed from $ONOO^-$ can react with nucleic acids, proteins, and lipids, resulting in damage to the cell membrane or intracellular organelles [4,5].

Maulik and co-workers [6] also showed that NO plays a significant role in transmembrane signaling in the ischaemic myocardium. This group suggested that myocardial NO signaling can be switched off due to inactivation of NO by reactive oxygen species (ROS) and were the first to suggest that ROS may negatively influence NO-cGMP signaling. Superoxide generated during ischaemia may quench NO and thus reduces NO

Abbreviations: AO, aortic output; cAMP, cyclic adinosine mono phosphate; cGMP, cyclic guanilyl mono phosphate; eNOS, endothelial nitric oxide synthase; LPO, lipid hydroperoxide; NO, nitric oxide; NOS, nitric oxide synthase; O_2^-, superoxide; $ONOO^-$, peroxynitrite; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; RPO, red palm oil; SFA, saturated fatty acids; SOD, superoxide dismutase

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levels that may be essential for normal cardiomyocyte signaling.

Cardiac stress adaptation is possibly jeopardized in hyperlipidaemia due to altered NO-cGMP pathway function in vascular and myocardial tissue. Szilvassy et al. [7] found that a cholesterol-enriched diet decreased both vascular NO and cGMP concentrations and increased O_2^- production. Several other studies have also shown that a high-cholesterol diet decreases cardiac NO concentration and impairs NO-cGMP signaling in both endothelium [8] and non-endothelial cells with a significant decrease in cardiac NO-concentrations [9,10]. Giricz et al. [11] found an increase in O_2^- production with decreased cardiac NO-levels in cholesterol-fed rats. Nitric oxide synthase (NOS) activity was unchanged but increased O_2^- production and subsequent NO quenching was responsible for the decreased NO-concentrations in the hyperlipidaemic myocardium. This concept is further supported by data showing that hyperlipidaemia stimulates $ONOO^-$ generation in the heart, which leads to subsequent myocardial dysfunction [12].

We have previously shown that dietary RPO-supplementation protects against the harmful consequences of myocardial ischaemia in rats fed a normal or cholesterol-enriched diet [14,15]. RPO contains a mixture of saturated fatty acids (SFAs) (51%), MUFAs (38%), polyunsaturated fatty acid (PUFAs) (11%) and antioxidants, tocopherols and tocotrienols [16]. These antioxidants can act as scavengers of damaging oxygen-free radicals [4,17–19]. Das and co-workers [20] showed that perfusion of the isolated heart with palm tocotrienol protected against the ischaemia/reperfusion injury by inhibition of cSrc activation and proteasome stabilization. Newaz and co-workers [21] showed an antioxidant protection by gamma-tocotrienols in hypertensive rats when compared with control animals. They suggested that the improved NOS activity in blood vessels and increased NO availability were mediated through the antioxidant properties of gamma-tocotrienol where it effectively scavenges the free radicals. Venditti and co-workers [22] also reported that vitamin E treatment offers protection against ischaemia/reperfusion-induced oxidative stress. However, the precise mechanism for these cardioprotective effects remains unknown.

Although previous studies have shown that tocotrienols per se are effective in protecting against oxidative stress, little is known about how RPO-supplementation protects against ischaemia/reperfusion injury, particularly when used as a supplement with a high-cholesterol diet. In the present study we investigated the possible mechanisms involved in the cardioprotective effects of RPO which contains lipid soluble anti-oxidants and fatty acids. Although this makes it difficult to ascribe the beneficial effects of RPO exclusively to either the fatty acids or lipid soluble anti-oxidants, we chose to focus on the potential effects of RPO on the myocardial NO-cGMP pathway.

The aims of this study were to determine whether RPO-supplementation influences: (1) myocardial susceptibility to ischaemia/reperfusion injury in hearts from animals on a normal or hypercholesterolaemic diet, (2) the function of the myocardial NO-cGMP signaling pathway, (3) enzymatic NOS and superoxide dismutase (SOD) activities, and (4) production of cardiac NO and lipid hydroperoxides (LPO).

2. Materials and methods

All animals received humane care in accordance with the Principle of Laboratory Animal Care of the National Society of Medical Research and the Guide for the Care and use of Laboratory Animals of the National Academy of Sciences (National Institutes of Health publications no. 80–23, revised 1978).

3. Experimental model

3.1. Experimental groups

Seven-week-old male Wistar rats were randomly allocated to 4 groups according to the dietary supplementation they received.

Group 1: Standard rat chow (control).

Group 2: Standard rat chow plus RPO (7 g RPO per kg diet).

Group 3: Standard rat chow, containing 2% cholesterol.

Group 4: Standard rat chow containing 2% cholesterol plus RPO (7 g RPO per kg diet).

The rats were fed a standard rat chow diet or 2% cholesterol-enriched diet [11] for 6 weeks. Rats from the control group consumed an average of 25 g food/day standard rat chow, containing 0.625 g fat, which provides 8.7% of the energy intake. Protein intake was 4.5 g (28% of energy intake). In two additional groups cholesterol-enriched and standard rat chow diet were supplemented with Carotino RPO-baking fat (7 g RPO per kg diet). There was a 21% increase in fat intake in the RPO-supplemented experimental groups. The red palm oil (RPO) baking fat added 70.0 μg carotenoids and 87.5 μg vitamin E (tocotrienols and tocopherols) to that present in the standard rat chow diet [16]. The standard rat chow was supplied by Atlas Animal Foods (Cape Town, South Africa) and regularly analyzed to monitor possible composition variations between batches. The respective energy and macronutrient content of the standard rat chow diet (control) and standard rat chow diet supplemented with RPO are indicated in Table 1.

Table 1
Approximate energy and macronutrient content of standard rat chow diet (control) and standard rat chow diet supplemented with red palm oil

Nutrient	Standard rat chow diet (control)[a]	Standard rat chow diet supplemented with red palm oil[b]
Energy (kJ)	272.5	277.6
Total non-structural carbohydrates (g)	8.375	8.375
Total protein (g)	4.5	4.5
Total fat (g)	0.625	0.758
Total SFA (g)	0.139	0.206
Total MUFA (g)	0.168	0.219
Total PUFA (g)	0.297	0.312
Total *n*−6 PUFA (g)	0.247	0.248
Total *n*−3 PUFA (g)	0.049	0.061

SFA (g) = saturated fatty acids.
MUFA (g) = monounsaturated fatty acids.
PUFA (g) = polyunsaturated fatty acids.
[a]25 g of standard rat chow per day.
[b]25 g of standard rat chow plus 0.175 g red palm oil per day.

3.2. Working heart perfusion

Rats weighing 300–400 g were anaesthetized with sodium thiopentone before the hearts were excised and placed in 4 °C Krebs–Henseleit buffer. Hearts were transferred to the standard working heart perfusion apparatus and perfused with a Krebs–Henseleit buffer equilibrated with 95% O_2 and 5% CO_2 at 37 °C (118.5 mM NaCI; 4.75 mM KCI; 1.2 mM $MgCI \cdot 6\ H_2O$; 1.36 mM $CaCI_2$; 25 mM $NaHCO_3$; 1.2 mM KH_2PO_4; 11.0 mM glucose) at a perfusion pressure of 100 cm H_2O.

The aorta was cannulated and retrograde perfusion initiated. During this initial perfusion in the Langendorff mode, the opening to the left atrium was cannulated.

Following a 5-min stabilization period in the Langendorff mode, hearts were switched to the working heart mode for 20 min. The temperature of both the perfusate and the air surrounding the heart, was thermostatically controlled and maintained at 37 °C. Hearts were then subjected to 25 min of total global ischaemia at 34 °C. At the end of ischaemia, hearts were reperfused in the Langendorff mode for 10 min, followed by 15 min working heart perfusion. In order to reduce the incidence of reperfusion arrhythmias, a 2% lignocaine solution was used for the initial three minutes of reperfusion of all hearts.

To assess myocardial NO- and cGMP concentrations, NOS- and SOD activities and LPO production, hearts were freeze-clamped with Wollenberger clamps pre-cooled in liquid nitrogen at the end of the pre-ischaemic working heart perfusion, after 10 min ischaemia and 10 min into reperfusion, and samples were stored at −80 °C until assays were performed.

3.3. Parameters measured and calculations used

3.3.1. Mechanical function parameters measured

In order to compare the functional recovery of the hearts in the different groups, the heart rate (beats/min), coronary flow (ml/min), aortic flow (ml/min) and left ventricular developed pressure (the difference between systolic- and diastolic pressure in mmHg) were monitored 25 min into the experiment and 25 min after reperfusion.

Coronary flow and aortic flow rates were measured at 5 min intervals. Aortic output (AO) recovery was calculated by dividing the AO measured after ischaemia by the value before ischaemia and expressing these values as a percentage.

3.4. Biochemical parameters measured

3.4.1. Measurement of cGMP

Myocardial cGMP concentrations were determined using radioimmunoassay kits obtained from Amersham Corp. (Amersham, UK). For cGMP assays, freeze-clamped hearts were freeze-dried before 20–25 mg of dry tissue was extracted in 5% trichloroacetic acid. The extracted sample was ether-washed three times for 5 min wash cycles. These samples were diluted 1:10 (V/V) and acetylated for the ^{125}I-labeled cGMP assay. The IC_{50} for the cGMP assay was 25 pmol/tube [24].

3.4.2. Measurement of myocardial nitrate/nitrite concentrations

An indirect estimate of myocardial NO concentrations was obtained by using a nitrate/nitrite assay kit (Cayman Chemicals, Cayman Islands), which measures total nitrate/nitrite concentration in the myocardial tissue sample. Approximately 200 mg of

frozen cardiac tissue was homogenized in 0.5 ml PBS (pH 7.4) and centrifuged at 10,000*g* for 20 min. Supernatant was ultra-filtered using a 30 kDa molecular weight cut-off filter (Millipore) and 40 μl of the filtrate was assayed.

3.4.3. NO in isolated cardiomyocytes

For direct measurement of intracellular NO production, cardiomyocytes from RPO-supplemented and control hearts were isolated by collagenase digestion, followed by incubation in a Krebs–Henseleit/2% bovine serum albumin buffer in the presence of 10 uM DAF-2/DA (0.5×10^6 cells/ml). Following incubation and washing, intracellular fluorescence of DAF-triazol (DAF-2 T, oxidized from DAF-DAF-2/DA) was analyzed by flow cytometry [23].

3.4.4. Measurement of cardiac nitric oxide synthase activity

Total NOS activity was measured using a NOS assay-kit which is based on biochemical conversion of L-arginine to L-citrulline by NOS (Cayman Chemicals, Cayman Islands). This NOS kit measures the conversion of the radioactive substrate [^{14}C] arginine to [^{14}C] citrulline and has a sensitivity to the picomole range. The NOS activity is quantified by counting the radioactivity in the eluate in a Beta-counter after performing the assay.

Approximately 100 mg of frozen cardiac tissue was homogenized in 0.5 ml homogenization buffer (1:10 dilution) and centrifuged at 10,000*g* for 15 min at 4 °C. For the assay 250 μl of supernatant was used.

3.4.5. Measurement of cardiac superoxide dismutase activity

Total activity of SOD was measured using a SOD kit (Cayman Chemicals, Cayman Islands) that utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthase. One unit of SOD is defined as the amount of enzyme required to exhibit 50% dismutation of the superoxide radical. The SOD assay measures all three types of SOD. Approximately 200 mg of frozen cardiac tissue was homogenized in 1.0 ml ice-cold HEPES buffer and centrifuged at 1500*g* for 5 min at 4 °C. For the assay we used 10 μl of the supernatant.

3.4.6. Measurement of cardiac lipid hydroperoxide production

LPO production in cardiac muscle was assessed using a LPO assay kit (Cayman Chemicals, Cayman Islands), which measures the hydroperoxides directly utilizing the redox reaction with ferrous ions. Hydroperoxides are highly unstable and react readily with ferrous ions to produce ferric ions. The resulting ferric ions are detected using thiocyanate ion as the chromagen.

Approximately 200 mg of frozen cardiac tissue was homogenised in 0.5 ml HPLC-grade water. An equal volume of Extract R saturated methanol was added and mixed thoroughly by vortexing. We then added 1.0 ml of cold chloroform, mixed thoroughly and centrifuged at 1500*g* for 5 min at 0 °C. The bottom chloroform layer was collected and 500 μl of this extract was used for the assay.

3.5. Statistical methods

Values are presented as mean ± SEM. Some values are presented as percentage change from the baseline values. To calculate the percentage change, the values were divided by baseline values and multiplied by 100. To adjust the *x*-axis to zero, 100 was subtracted from the values obtained. One way ANOVA with Bonferroni's post correction was used to measure significance. $P < 0.05$ was considered significant.

4. Results

4.1. Cardiac mechanical function in the isolated pefused rat hearts

In order to compare the functional recovery of the hearts from the different groups, the heart rate (beats/min), coronary flow (ml/min), aortic flow (ml/min) and left ventricular developed pressure (mmHg) were monitored after 25 min perfusion and 25 min after reperfusion (Table 2). There were no significant differences between the groups for any parameters measured before ischaemia. These data suggests that RPO-, cholesterol and cholesterol/RPO-supplementation did not affect basal, normoxic cardiac function. Reperfusion AO values were however decreased during reperfusion (Table 2).

4.2. Reperfusion cardiac mechanical function

The AO recovery of RPO-supplemented hearts was higher than in the control hearts, suggesting that RPO protected these hearts against ischaemia/reperfusion injury (72.1 ± 3.2% versus 54.0 ± 3.2%, $P < 0.05$) (Fig. 1). Similarly, the AO recovery of hearts from rats in the cholesterol/RPO-supplemented group was significantly increased when compared with hearts from the cholesterol-fed group (64.7 ± 2.4% versus 42.9 ± 6.3%, $P < 0.05$). These values were however decreased when compared with AO recovery of hearts of the RPO-supplemented control group (72.1 ± 3.2% versus 64.7 ± 2.4%, $P < 0.05$) (Fig. 1).

4.3. Cardiac cGMP concentrations

Baseline cGMP concentrations refer to the values 25 min into the experiment, before ischaemia was induced

Table 2
Cardiac functional parameters in the 4 experimental groups before and after ischaemia

Time Point	Group	HR	CF	AO	LVDevP
20 min perfusion	Control	234.77±22.00	21.57±1.90	48.14±2.83	127.67±4.21
	RPO	258.59±17.88	20.43±1.38	46.43±3.15	128.59±4.81
	Chol	236.50±14.95	21.00±1.73	51.25±3.74	143.63±11.95
	Chol/RPO	222.16±16.65	16.20±1.53	47.23±1.61	131.34±7.30
25 min reperfusion	Control	240.31±17.06	22.29±1.71	28.57±2.88*	101.1±12.62
	RPO	265.83±17.79	21.29±1.91	33.43±2.50	112.84±6.05
	Chol	251.50±1.89	23.00±3.61	23.62±4.14*	132.83±7.05
	Chol/RPO	227.72±19.82	17.40±1.99	31.31±1.79*	131.36±6.42

Working heart perfusion cardiac functional parameters of control and cholesterol-fed animals with/without 6 weeks red palm oil-supplementation for 6 weeks. Heart rate (HR, beats/min); Coronary flow (CF, ml/min); Aortic output (AO, ml/min): Left ventricular developed pressure (LVDevP). Values are mean±SEM ($n = 7$ in each group)
*$P < 0.05$ vs. pre-ischaemic values.

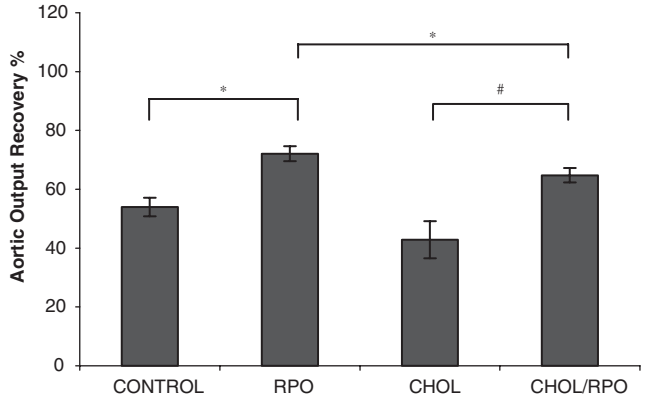

Fig. 1. Aortic output recovery of hearts from rats fed a standard rat chow diet (control) or a standard rat chow plus cholesterol diet and/or RPO supplementation 25 min into reperfusion ($n = 7$ per group) (*$P < 0.05$ for the group versus the control group) (#$P < 0.05$ for the group versus the cholesterol group) (mean±SEM).

(control: 21.6±2.4 pmol/g; RPO: 25.4±4.0 pmol/g; cholesterol: 26.1±2.8 pmol/g and cholesterol/RPO: 36.4±9.1 pmol/g). All values in Fig. 2 represent the percentage change in cGMP concentrations from the baseline value at 10 and 25 min ischaemia and 10 min into reperfusion. The cGMP concentrations were significantly increased in the RPO-supplemented hearts when compared with control hearts 10 min into ischaemia (RPO: 132.0±36.3% versus control: 42.7±24.4%, $P < 0.05$)($n = 5$ per group per time point).

4.4. Cardiac nitrate/nitrite oxide concentrations

Baseline (pre-ischaemic), ischaemic and reperfusion NO concentrations were similar in all groups at all time points investigated (Fig. 3).

4.5. Cardiomyocyte NO levels

However, with direct intracellular NO detection in isolated rat cardiomyocytes we found an increase in NO production after 120 min of simulated ischaemia (hypoxia) in the RPO-supplemented group when compared with baseline values in RPO-supplemented group and hypoxic control group (2.9±0.1 arbitrary units versus 2.5±0.1 arbitrary units and 2.4±0.1 arbitrary units, respectively, $P < 0.05$) (Fig. 4A). The cholesterol/RPO-supplemented group showed a similar increase in NO production after 120 min of simulated ischaemia (hypoxia) when compared with baseline NO production in this group (2.5±0.1 arbitrary units versus 2.1±0.1 arbitrary units, $P < 0.05$) (Fig. 4B).

4.6. Cardiac nitric oxide synthase activity

Baseline myocardial NOS activity was unchanged for cholesterol-, cholesterol/RPO- and RPO-supplemented groups. However, myocardial NOS activity was increased in cholesterol/RPO-supplemented group when compared with cholesterol group at 10 min ischaemia (6.8±0.6% versus 4.5±0.6%, $P < 0.05$) ($n = 5$ per group per time point).

Myocardial NOS activity was decreased at 10 min ischaemia in the control group when compared with baseline values for this group (5.7±0.3% versus 3.9±0.5%, $P < 0.05$)($n = 5$ per group per time point) (Fig. 5).

4.7. Cardiac superoxide dismutase activity

Baseline- (20 min perfusion), ischaemic- and reperfusion SOD activity was similar in all groups at all time points investigated (Table 3).

4.8. Cardiac lipid hydroperoxide production

Direct measurement of myocardial hydroperoxides in rats fed a standard rat chow or a standard rat chow plus cholesterol and/or RPO-supplementation showed no significant difference in baseline-, ischaemic- and

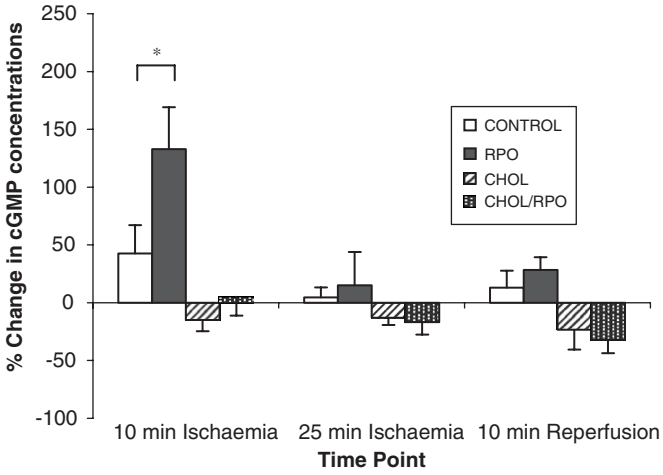

Fig. 2. Percentage change in myocardial cGMP concentrations in the 4 experimental groups from the baseline value at 10 and 25 min ischaemia and 10 min into reperfusion ($n = 5$ per group per time point) (*$P < 0.05$ for the group versus the control group) (mean ± SEM).

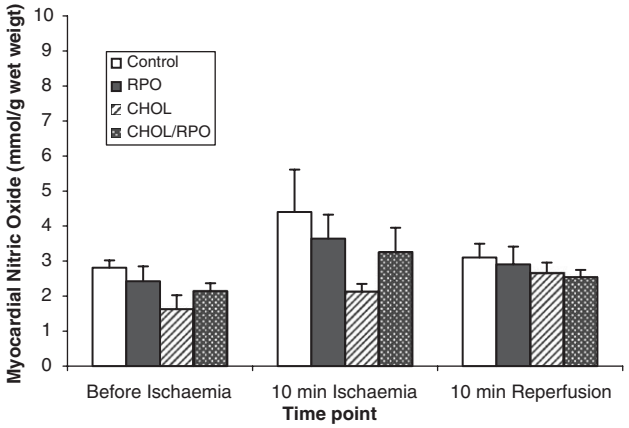

Fig. 3. Total myocardial NO concentrations in the 4 experimental groups before ischaemia, during ischaemia and during reperfusion (*$P < 0.05$ for the group versus the control group) ($n = 5$ per group per time point) (mean ± SEM).

reperfusion hydroperoxide concentrations when compared with the control group (Table 3).

5. Discussion

We found that RPO-supplementation of a standard rat chow (control) or cholesterol-enriched diet improves AO recovery after an ischaemic event. The improved functional recovery in hearts from rats on a control diet supplemented with RPO was associated with an elevation in myocardial cGMP concentrations during ischaemia. RPO supplementation increased hypoxic (simulated ischaemia) intracellular NO production in isolated cardiomyocytes from control diet fed rats. Hypoxia only increased cardiomyocyte NO levels in control, and cholesterol diet fed rats in the presence of RPO supplementation. Myocardial NOS activity decreased during ischaemia in the hearts from control rats, but RPO-supplemention of the cholesterol-fed group increased myocardial NOS activity during ischaemia.

5.1. Effects of cholesterol-enriched diet on baseline myocardial NO concentrations

Onody and co-workers [12] showed that a cholesterol-enriched diet reduces cardiac NO concentrations, enhances cardiac formation of O_2^- and stimulates $ONOO^-$ generation in the heart, which leads to myocardial dysfunction. They speculated that the decreased myocardial NO concentrations were not due to impaired NO synthesis, but was possibly caused by increased breakdown of cardiac NO due to hyperlipidaemia [11,25]. Girics and co-workers [11] propose that increased O_2^- production in hyperlipidemic hearts would promote the reaction of this ROS with NO to form $ONOO^-$ and supports the concept that elevated ROS production is responsible for decreased NO concentrations in the hyperlipidemic myocardium.

5.2. NO production during ischaemia

Our results showed that myocardial NOS activity was impaired under ischaemic conditions in control hearts when compared with baseline (non-ischaemic) values of the same group. These data are in agreement with other studies, which showed decreased eNOS activity during ischaemia with only partial restoration upon reperfusion [26]. Surprisingly, ischaemic myocardial NOS activity of hearts from the cholesterol/RPO-group was increased when compared with the cholesterol group. These findings are supported by the data obtained with the isolated cardiomyocytes. The cardiomyocytes of rats supplemented with cholesterol/RPO showed an increased intracellular cardiomyocyte NO production

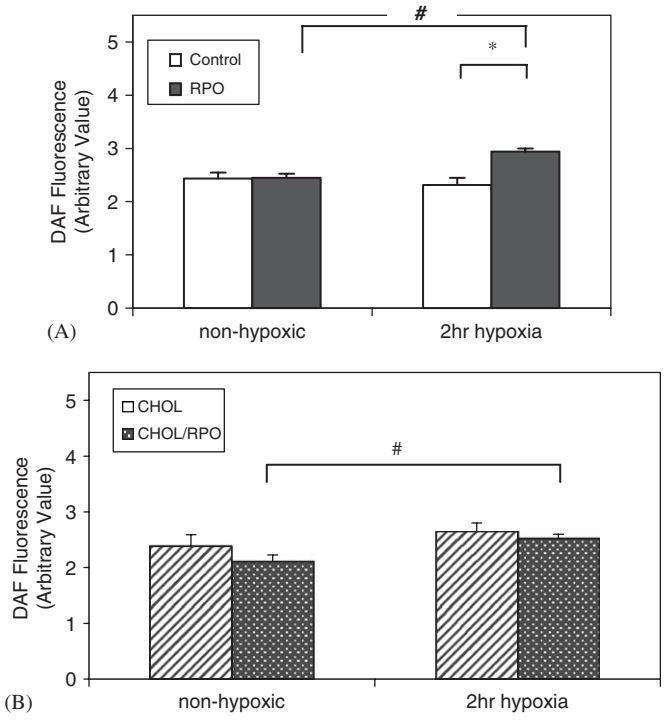

Fig. 4. Intracellular nitric oxide as detected by DAF fluorescence in isolated cardiomyocytes. (A) (*$P<0.05$ for the group versus the control group) (#$P<0.05$ for the hypoxic group versus the non-hypoxic group) ($n=6$ per group) (B) (#$P<0.05$ for the hypoxic group versus the non-hypoxic group) ($n=6$ per group). Experimental groups consist of samples from different hearts (mean ± SEM).

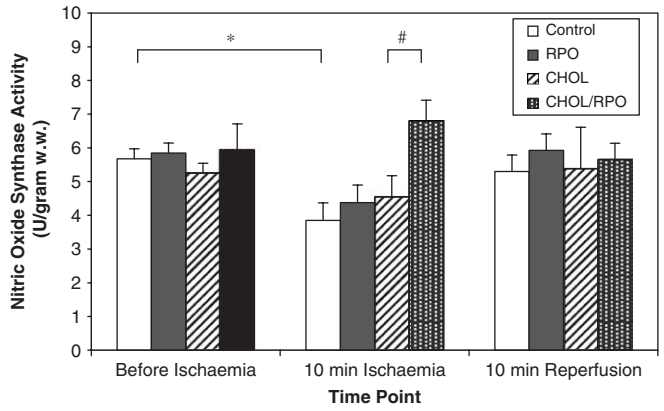

Fig. 5. Myocardial NOS activity in the 4 experimental groups before ischaemia, during ischaemia and in reperfusion ($n=5$ per group per time point)(*$P<0.05$ for the group versus the same group before ischaemia) (#$P<0.05$ for the group versus the cholesterol group) (mean ± SEM).

after 120 min of simulated ischaemia (hypoxia) when compared with baseline NO-concentration in cholesterol/RPO-supplemented group. Newaz et al. [21] demonstrated antioxidant-mediated protection by gamma-tocotrienols in hypertensive rats. This group suggested that improved NOS activity in blood vessels and increased NO availability were mediated through the antioxidant properties of gamma-tocotrienol, which effectively scavenges the free radicals. Previous studies [12] have demonstrated that a cholesterol-enriched diet increased basal myocardial superoxide generation. Based on these data we speculate that free radical generation would be further increased during ischaemia. Elevated concentrations of free radicals may contribute to the suppressed NOS activity in the ischaemic hearts of the cholesterol-fed rats. The inclusion of RPO, containing tocopherols and tocotrienols, could potentially improve NOS activity in the cholesterol/RPO-supplemented group by reducing free-radical-induced NOS enzyme damage.

Although there appears to be a discrepancy between ischaemic NOS activity and NO concentrations in the hearts, these data may be explained by the findings of Zweier and co-workers. These authors have shown

Table 3
Baseline (20-minute perfusion), ischaemic and post-ischaemic myocardial superoxide dismutase activity and lipid hydroperoxide levels in the 4 experimental groups ($n = 5$ per group per time point)

	Group	20 min perfusion	10 min ischaemia	10 min reperfusion
SOD (U/ml)	Control	1.55±0.22	1.90±0.37	1.92±0.10
	RPO	1.77±0.35	1.61±0.14	2.05±0.23
	Chol	2.31±0.26	2.03±0.28	1.71±0.26
	Chol/RPO	1.93±0.19	2.02±0.32	2.42±0.09
LPO (μmol/l)	Control	10.44±0.36	11.93±0.79	10.14±0.18
	RPO	9.18±0.51	9.49±0.73	9.44±0.22
	Chol	9.45±0.52	9.72±0.67	9.25±1.38
	Chol/RPO	9.63±1.00	9.50±1.24	10.09±1.82

SOD = superoxide dismutase.
LPO = lipid hydroperoxide (values are mean±SEM).

that NO generation from nitrite via a NOS-independent pathway can be the major source of NO during ischaemia [27].

5.3. RPO-supplementation and NO-cGMP signaling

Improved functional recovery of hearts of rats fed a diet supplemented with RPO when compared with control rats was associated with elevated cGMP concentrations early in ischaemia. Increased intracellular cardiomyocyte NO concentrations as observed in the RPO-supplemented group after 120-min hypoxia may contribute to the elevated cGMP concentration and may confer some of the cardioprotection to the ischaemic/reperfused heart. Maulik et al. [6] showed that NO plays a significant role in transmembrane signaling in the ischaemic myocardium. The signaling seems to be transmitted via cGMP and they suggested that NO signaling is switched on- and off due to inactivation of NO by ROS. Therefore, if dietary RPO containing vitamin E antioxidants act as free radical scavengers, elevated cGMP concentrations early in ischaemia, through increased NO signaling, could be responsible for improved functional recovery in hearts of rats supplemented with RPO versus non-RPO fed hearts in the control group.

5.4. Dietary vitamin E and generation of NO, O_2^- and $ONOO^-$ in cholesterol-enriched diets

Our data indicate that RPO-supplementation of a high-cholesterol diet improved functional recovery of the ischaemic/reperfused heart. Despite the increased intracellular cardiomyocyte NO concentrations observed in the cholesterol/RPO-supplemented group after 120-min hypoxia when compared with the non-hypoxic cholesterol/RPO-supplemented group, this improvement could not be linked with increased myocardial GMP levels. Previous studies have shown that cholesterol-enriched diets impair NO-cGMP signaling in both endothelial and non-endothelial cells [7–10]. Therefore, based on our results we propose that the protective effect of RPO in high-cholesterol diets may be due to the dietary RPO antioxidant characteristics. Vitamin E acts as a free radical scavenger that can react with oxygen, superoxide anion radical and hydroxyl radical [28].

Chow and Hong [29] reported that dietary vitamin E is capable of reducing the production and/or availability of not only O_2^-, but also NO and $ONOO^-$. By reducing available O_2^- and NO, vitamin E may alleviate nitric toxicity by reduced formation of reactive $ONOO^-$.

However, it is not clear if the action of vitamin E to reduce the generation of O_2^- and other ROS is independent of its antioxidant function.

5.5. Effects of diets rich in PUFAs and SFAs in cardiovascular disease

In a previous study we reported an increase in baseline myocardial total % SFA composition of total phospholipid with RPO-supplementation of the control and cholesterol groups, respectively [15].

Diniz et al. [13] reported that changes in dietary fatty acids affect cardiac oxidative stress. They showed that diets rich in PUFAs, despite the beneficial effects on serum lipids, were deleterious when compared with SFAs in the heart by increasing cardiac susceptibility to lipid-peroxidation. Their observation that SFA-fed rats had lower myocardial hydroperoxide concentrations than PUFA-fed rats demonstrates the importance of the PUFA: SFA ratio on lipid-peroxidation and the level of antioxidants. They also showed that PUFA rats had diminished SOD activity.

We have also reported in a previous study that RPO-supplementation of rats on a standard rat chow diet (control) increased total myocardial PUFAs over the 25-min period of ischaemia [15]. Bagchi and Puri [30] reported that vitamin E protects PUFAs in cell membranes from peroxidation, which may confer some

of the cardioprotection in RPO-supplemented control and cholesterol-treated rats.

5.6. Ischaemia versus reperfusion

In the current study our results suggest that the beneficial changes in cGMP concentrations, NOS activity and myocardial NO production occurs during the ischaemic period. During reperfusion we could not find any significant difference in the intermediates of this signaling pathway when comparing the groups. This may suggest that the RPO-effect on the NO-cGMP pathway is restricted to the ischaemic period. In a recent study done in our laboratory [31], the results suggest that dietary RPO-supplementation activates/deactivates MAPKs, PKB/Akt and caspase during reperfusion with consequential improved functional recovery. This argues for the involvement of two mechanisms for RPO induced protection, one via the NO-cGMP pathway during ischaemia and the other via the MAPKs, PKB/Akt and caspase during reperfusion. These findings create opportunities for further investigations to elucidate RPO-mediated mechanisms involved in cardiac protection.

5.7. Limitations of the study

The cocktail of anti-oxidants and fatty acids contained in RPO preclude us from determining which specific anti-oxidants or fatty acids are responsible for the cardioprotective effects of RPO. However, we show that RPO was able to protect the heart against oxidative stress even when used in relatively small dosages. Some may criticize our choice of dosages, but it allowed us to show that small dosages can provide potent protection during long-term supplementation, even in the presence of a harmful diet (cholesterol-fed group).

6. Conclusions

Our data show that RPO-supplementation improves AO recovery in hearts from both standard rat chow (control) and cholesterol-fed animals. The improved functional recoveries seen in control diet fed, RPO-supplemented hearts may be due to preservation of ischaemic myocardial NO and cGMP concentrations. However, RPO-supplementation of a high cholesterol diet probably protects the isolated rat heart against ischaemia/reperfusion injury by mechanisms independent of the NO-cGMP signaling pathway. This following sequence of events should be considered: In the presence of cholesterol, superoxide production is increased. Superoxide would compete with guanylyl cyclase for NO. NO could therefore contribute to lipid peroxidation, instead of cGMP production. Our results indicate that neither SOD activity, nor LPO production increased. It is thus tempting to speculate that RPO may have scavenged superoxide before it could be used to produce peroxynitrite and its breakdown products. This potentially improved NO availability for NO-cGMP signaling . However, we have no data to support this argument, but this possibility will be investigated in future.

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