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# p38-MAPK and PKB/Akt, possible role players in red palm oil-induced protection of the isolated perfused rat heart?

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**Abstract**

It has been shown that dietary red palm oil (RPO) supplementation improves reperfusion function. However, no exact protective cellular mechanisms have been established. To determine a potential mechanism for functional improvement, we examined the regulation of both mitogen-activated protein kinases (MAPKs) and PKB/Akt in the presence and absence of dietary RPO supplementation in ischemia/reperfusion-induced injury. Wistar rats were fed a control diet or control diet plus 7 g RPO/kg diet for 6 weeks. Hearts were excised and mounted on an isolated working heart perfusion apparatus. Cardiac function was measured before and after hearts were subjected to 25 min of total global ischemia. Hearts subjected to the same conditions were freeze clamped and used to characterize the degree of phosphorylation of extracellular signal-regulated kinase, p38, c-Jun $NH_2$-terminal protein kinase (JNK) and PKB/Akt. Dietary RPO supplementation significantly improved aortic output recovery ($72.1 \pm 3.2\%$ vs. $54.0 \pm 3.2\%$, $P < .05$). This improved aortic output recovery was associated with significant increases in p38 and PKB/Akt phosphorylation during reperfusion when compared with control hearts. Furthermore, a significant decrease in JNK phosphorylation and attenuation of poly(ADP-ribose) polymerase cleavage occurred in the RPO-supplemented group during reperfusion. Our results suggest that dietary RPO supplementation caused differential phosphorylation of the MAPKs and PKB/Akt during ischemia/reperfusion-induced injury. These changes in phosphorylation were associated with improved functional recovery and reduced cleavage of an apoptotic marker, arguing that dietary RPO supplementation may confer protection via the MAPK and PKB/Akt signaling pathways during ischemia/reperfusion-induced injury.


*Keywords:* Red palm oil; MAPKs; PKB; Ischemia; Reperfusion

## 1. Introduction

Cardiovascular disease remains one of the major causes of death in modern society. Although it was previously shown that dietary red palm oil (RPO) supplementation protects against global ischemia/reperfusion injury in the isolated perfused rat heart [1], the mechanism of action of RPO remains to be elucidated.

Several signal transduction pathways in the heart are regulated in direct response to ischemia/reperfusion-induced injury. One of the best-characterized signal transduction pathways in the heart is the family of mitogen-activated protein kinases (MAPKs). The MAPKs are a family of serine–threonine kinases that are activated in response to a variety of extracellular stimuli [2,3]. Three major MAPKs, including extracellular signal-regulated protein kinase (ERK), p38 and c-Jun $NH_2$-terminal protein kinase (JNK), have been implicated in the response to ischemia and reperfusion in the heart [4,5]. All three MAPKs have been shown to play pivotal roles in transmission of signals from cell surface receptors to the nucleus and are involved in cell growth, differentiation and apoptosis [6–8]. Another potential target of RPO might be the serine–threonine kinase PKB/Akt. PKB/Akt contains a pleckstrin homology (PH) domain that is part of a slightly larger portion in the $NH_2$ terminus called the Akt homology domain. The phosphoinositide 3-kinase (PI3-K) product phosphatidyli-

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nositol-3,4-bisphosphate bind in vitro directly to the PH domain and increases enzyme activity [9]. PKB/Akt has been shown to be activated by factors that stimulate PI3-K, including thrombin, platelet-derived growth factor and insulin [9]. There is also increasing evidence that the PKB/Akt pathway participates in ischemia/reperfusion-induced injury [10,11].

Very little or any information regarding the effects of fatty acids and antioxidants (major components of RPO) on the MAPK family and PKB is available in the heart. Chen et al. reported that eicosapentaenoic acid inhibits hypoxia-reoxygenation-induced injury by attenuation of p38 MAPK [12]. Furthermore, it was reported that antioxidant treatment of myocytes suppressed the increase in ROS and blocked ERK activation and the subsequent cardiac hypertrophy induced by these stimuli [13].

However, as far as we know, no evidence exists for an interaction between RPO and the activation/inhibition of the MAPKs, and the prosurvival kinase PKB during ischemia and reperfusion. In order to assess the possible mechanisms of protection, the isolated perfused rat heart model was used to determine whether dietary RPO supplementation was associated with changes in the regulation of the MAPKs and PKB/Akt during ischemia and reperfusion.

## 2. Materials and methods

### 2.1. Antibodies and chemicals

Antibodies were purchased from Cell Signalling Technology and all other chemicals were obtained from Sigma (St. Louis, MO).

### 2.2. Experimental model

All animals received humane care in accordance with the *Principles 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). Male Wistar rats were divided into two groups: a control group receiving normal rat chow and an experimental group receiving normal chow plus 7 g RPO/kg chow for 6 weeks. The approximate energy and macronutrient content of the two diets are indicated in Table 1. The rat chow was supplied by Atlas Animal Foods, Cape Town, South Africa, and regularly analyzed to monitor possible variations between batches. The rats consumed an average of 25 g food/day, the standard rat chow, containing 0.625 g of fat, which provides 8.7% of the energy intake. Protein intake was 4.5 g (28% of energy intake). In the experimental group, 0.175 g of RPO baking fat was supplemented every morning, for 6 weeks, before they received their daily allowance of rat chow. Thus, there was a 21% increase in fat intake in the RPO-supplemented experimental group. The RPO used in this study provided 70 μg of carotenoids and 87.5 μg of vitamin E (tocopherols and tocotrienols) [14], additional to any other antioxidants present in the standard rat chow diet (antioxidant nutrient status of control rat chow not provided by supplier due to confidentiality).

Rats weighing 300 to 400 g were anaesthetized with sodium thiopentone before the hearts were rapidly excised and placed in ice-cold Krebs–Henseleit buffer. Hearts were transferred to the standard working heart perfusion apparatus and perfused with a Krebs–Henseleit buffer, pH 7.4, containing (in mM) NaCl 119, $NaHCO_3$ 24.9, KCl 4.74, $KH_2PO_4$ 1.19, $MgSO_4$ 0.6, $Na_2SO_4$ 0.59, $CaCl_2$ 1.25 and glucose 10. The buffer was oxygenated and kept at pH 7.4 by gassing with 95% $O_2$/5% $CO_2$. The aorta was cannulated and retrograde perfusion was 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 checked at regular intervals to ensure that the temperature was maintained at 37°C irrespective of coronary flow. Hearts were then subjected to 25 min of total

Table 1
Approximate energy and macronutrient contents of rat diets

| Nutrient | Standard rat chow diet (control)* | Standard rat chow diet supplemented with RPO** |
|---|---|---|
| Energy (kJ) | 272.5 | 277.6 |
| Total nonstructural 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.
* Twenty-five grams of standard rat chow per day.
** Twenty-five grams of standard rat chow plus 0.175 g of red palm oil per day.

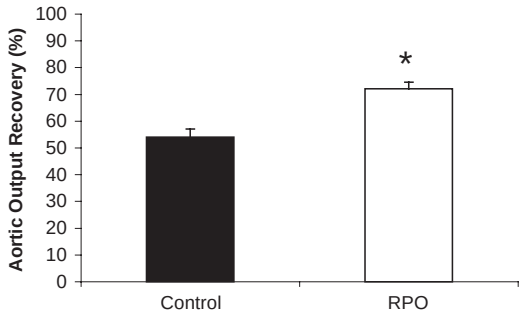


Fig. 1. The percentage aortic output recovery of RPO-supplemented hearts vs. control hearts. Results are expressed as means±S.E.M. for seven independent experiments ($n=7$). RPO vs. control (*$P<.01$).

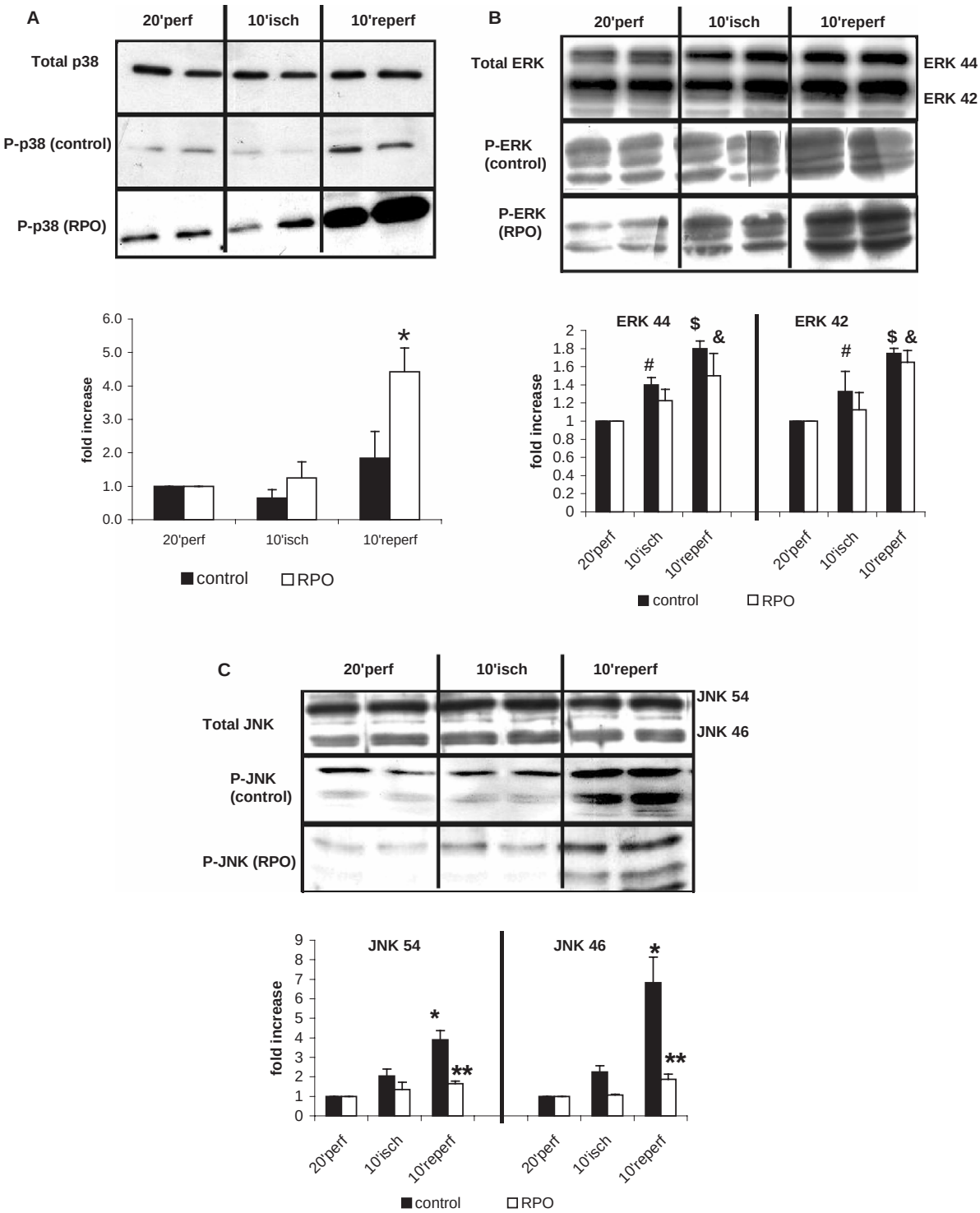


Fig. 2. (A) The effect of dietary RPO supplementation on p38 phosphorylation in hearts subjected to ischemia and reperfusion. Samples were analyzed by Western blotting with phospho-specific antibodies recognizing dual phosphorylated MAPKs. Results are expressed as means±S.E.M. for four independent experiments ($n=4$). 10′ reperf RPO vs. 10′ reperf control (*$P<.001$). (B) The effect of dietary RPO supplementation on ERK phosphorylation in hearts subjected to ischemia and reperfusion. Samples were analyzed by Western blotting with phospho-specific antibodies recognizing dual phosphorylated MAPKs. Results are expressed as means±S.E.M. for four independent experiments ($n=4$). ERK44: 10′ isch control vs. 20′ perf control ($^{\#}P<.01$); 10′ reperf control vs. 10′ isch control ($^{\$}P<.01$); 10′ reperf RPO vs. 20′ perf RPO ($^{\&}P<.001$). ERK42: 10′ isch control vs. 20′ perf control ($^{\#}P<.05$); 10′ reperf control vs. 10′ isch control ($^{\$}P<.01$); 10′ reperf RPO vs. 20′ perf RPO ($^{\&}P<.001$). (C) The effect of dietary RPO supplementation on JNK phosphorylation in hearts subjected to ischemia and reperfusion. Samples were analyzed by Western blotting with phospho-specific antibodies recognizing dual phosphorylated MAPKs. Results are expressed as means±S.E.M. for four independent experiments ($n=4$). JNK54: 10′ reperf RPO vs. 10′ reperf control (**$P<.001$). JNK46: 10′ reperf control vs. 20′ perf control (*$P<.001$); 10′ reperf RPO vs. 10′ reperf control (**$P<.001$).

global ischemia. At the end of ischemia, 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, 2% lignocaine solution was used for the initial 3 min of reperfusion of all hearts. To assess myocardial MAPKs activity, hearts were freeze clamped with Wollenberger clamps precooled in liquid nitrogen at the end of the preischemic working heart perfusion ($n$=4/group), after 10 min ischemia ($n$=4/group) and 10 min into reperfusion ($n$=4/group), and samples were stored at −80 °C.

### 2.3. Functional parameters measured

Coronary and aortic flow rates were measured by collecting 1-min samples of the respective effluents at 25 min into the preischemic perfusion and 25 min into reperfusion. Aortic output recovery was calculated by dividing the AO measured after ischemia by that measured before ischemia and expressing these values as percentage recovery.

### 2.4. Western blot analysis

Cardiac MAPKs and PKB/Akt as well as caspase-3 and poly(ADP-ribose) polymerase (PARP) protein were extracted with a lysis buffer containing (in mM): Tris 20, *p*-nitrophenylphosphate 20, EGTA 1, NaF 50, sodium orthovanadate 0.1, phenylmethyl sulfonyl fluoride (PMSF) 1, dithiothreitol (DTT) 1, aprotinin 10 μg/ml, leupeptin 10 μg/ml. The tissue lysates were diluted in Laemmli sample buffer, boiled for 5 min and 10 μg (MAPKs and PKB/Akt) or 50 μg protein (caspase-3 and PARP) were separated by 10% PAGE-SDS gel electrophoresis. The lysate protein content was determined using the Bradford technique [15]. The separated proteins were transferred to a PVDF membrane (Immobilon P, Millipore). These membranes were routinely stained with Ponceau Red for visualization of proteins. Nonspecific binding sites on the membranes were blocked with 5% fat-free milk in Tris-buffered saline — 0.1% Tween 20 (TBST) and then incubated with the primary antibodies that recognize phospho-specific ERK p42/p44 ($Thr^{202}/Tyr^{204}$), p38-MAPK ($Thr^{180}/Tyr^{182}$), JNK p54/p46 ($Thr^{183}/Tyr^{185}$), PKB ($Ser^{473}$ and $Thr^{308}$), caspase-3 (p17 fragment pAb) and PARP (p85 fragment pAb). Membranes were subsequently washed with large volumes of TBST (5×5 min) and the immobilized antibody conjugated with a diluted horseradish peroxidase-labeled secondary antibody (Amersham LIFE SCIENCE). After thorough washing with TBST, membranes were covered with ECL detection reagents and quickly exposed to an autoradiography film (Hyperfilm ECL, RPN 2103) to detect light emission through a non-radioactive method (ECL Western blotting). Films were densitometrically analyzed (UN-SCAN-IT, Silkscience) and phosphorylated protein values were corrected for minor differences in protein loading, if required. Experiments were performed (data not shown) to ensure that all signals were within the linear range of detection on the autoradiographs under our assay- and gel-loading conditions.

### 2.5. Data analysis

Data are presented as mean±S.E.M. Statistical significance was determined in multiple comparisons among independent groups of data in which ANOVA and the Bonferroni's post hoc test indicated significant differences. A $P$ value <.05 was considered statistically significant.

## 3. Results

### 3.1. Aortic output recovery

We used aortic output recovery as an indirect index of the severity of ischemia/reperfusion injury. These data suggest that RPO protected against the consequences of ischemia/reperfusion (72.1±3.2% vs. 54.0±3.2%, $P$<.05) (Fig. 1).

### 3.2. The effect to RPO supplementation on the phosphorylation of p38, JNK and ERK in hearts subjected to ischemia and reperfusion

Phosphorylation of p38, JNK (p46/p54-MAPK) and ERK 1/2 (p42/p44-MAPK) was determined by Western blotting using phospho-specific antibodies. As shown in Fig. 1A, p38 phosphorylation was significantly increased in the RPO-supplemented group during reperfusion vs. the control (perfusion) group (1.84±0.39- to 4.42±0.35-fold, $P$<.001). Ischemia/reperfusion caused significant increases

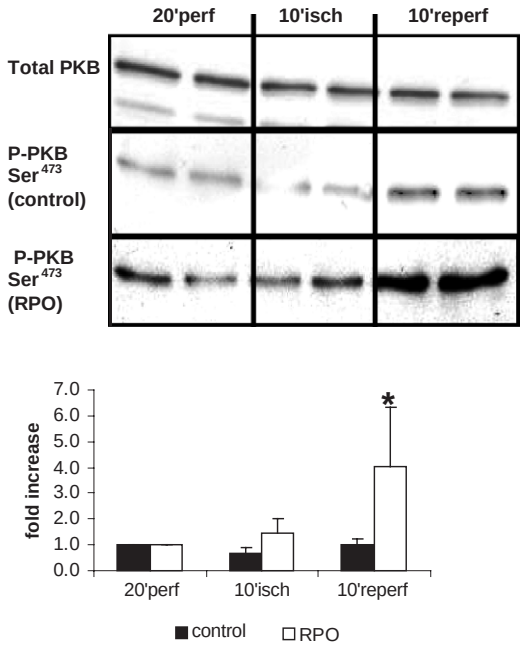


Fig. 3. The effect of dietary RPO supplementation on ERK phosphorylation in hearts subjected to ischemia and reperfusion. Samples were analyzed by Western blotting with phospho-specific antibodies recognizing dual phosphorylated MAPKs. Results are expressed as means±S.E.M. for four independent experiments ($n$=4). 10′ reperf RPO vs. 10′ reperf control (*$P$<.01).

in both JNK54 and JNK46 phosphorylation from 20′ perf to 10′ reperf (3.9±0.23-fold, $P<.001$, for JNK54 and 6.83±0.66-fold, $P<.001$, for JNK46). Dietary RPO supplementation caused a significant decrease in phosphorylation of both JNK54 and JNK46 (3.9±0.23- to 1.65±0.06-fold, $P<.001$, for JNK54 and 6.83±0.66- to 1.87±0.13-fold, $P<.001$, for JNK46). Although there were significant increases in both ERK44 and ERK42 phosphorylation during ischemia and reperfusion ($P<.001$ for both isoforms), this phosphorylation was unaffected by RPO supplementation (Fig. 2A–C).

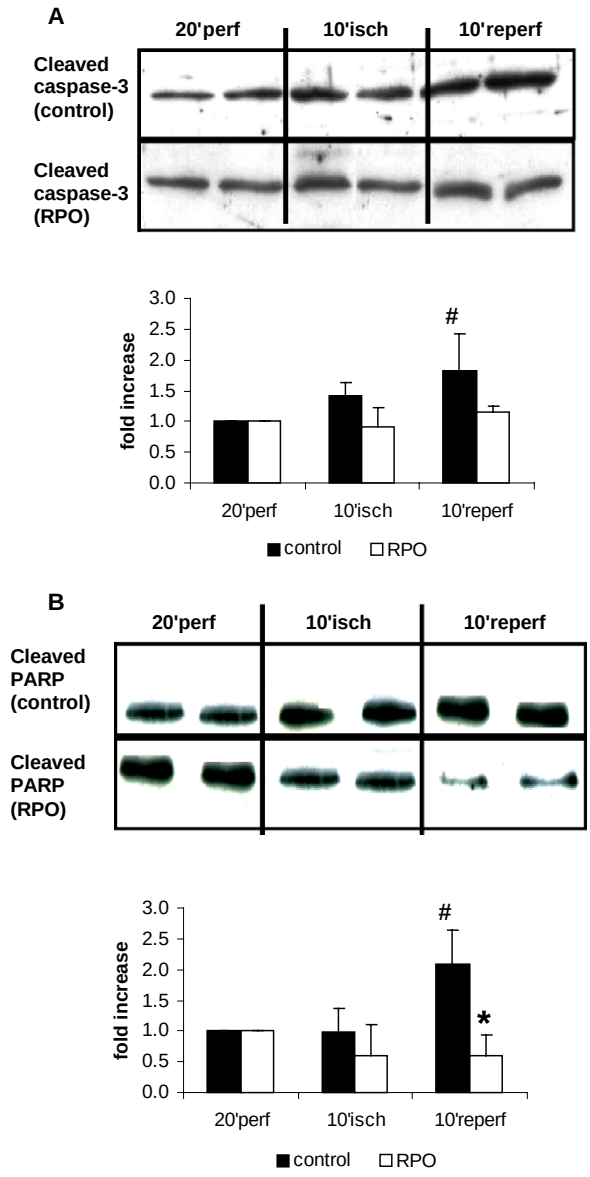


Fig. 4. (A, B) The effect of dietary RPO supplementation on caspase-3 activation and PARP cleavage during ischemia and reperfusion. Samples were analyzed by Western blotting with antibodies recognizing cleaved PARP and caspase-3. Results are expressed as means±S.E.M. for four independent experiments ($n=4$). Caspase-3: 10′ reperf control vs. 20′ perf control ($^{*}P<.05$). PARP: 10′ reperf control vs. 20′ perf control ($^{\#}P<.01$); 10′ reperf RPO vs. 10′ reperf control ($^{*}P<.001$).

### *3.3. The effect to RPO supplementation on the phosphorylation of PKB/Akt in hearts subjected to ischemia and reperfusion*

Phosphorylation of PKB/Akt ($Ser^{473}$) was determined by Western blotting using phospho-specific antibodies. There was a significant increase in PKB/Akt phosphorylation in the RPO-supplemented group compared to the control group during reperfusion (1.03±0.11- to 4.03±1.1-fold, $P<.01$) (Fig. 3).

### *3.4. The effect to RPO supplementation on caspase-3 activation and PARP cleavage in hearts subjected to ischemia and reperfusion*

The control group showed a significant increase in PARP cleavage during reperfusion compared to the perfusion group (2.1±0.27-fold, $P<.01$). However, RPO supplementation significantly attenuated PARP cleavage (2.1±0.27- to 0.6±0.17-fold, $P<.001$) during reperfusion. Although there was a significant increase in caspase-3 activation during reperfusion compared to perfusion in the control group ($P<.05$), RPO failed to attenuate this activation (Fig. 4A and B).

## 4. Discussion

We have demonstrated that dietary RPO supplementation offered significant protection against ischemia/reperfusion-induced injury in the isolated perfused working heart as reflected by improving functional recovery. Although no evidence exists for the role of RPO in functional recovery after ischemia/reperfusion-induced injury (except for our own [1]), some evidence does exist for an effect of some of the major components of RPO on cardiac function. For example, Meehan and Higgins [16] demonstrated that oleic acid improved functional recovery in ischemic/reperfused rat hearts. On the other hand, Serbinova et al. [17] showed that RPO vitamin E was more effective than tocopherols in protecting against ischemia/reperfusion injury in the isolated Langendorff perfused heart. Das et al. [18] also demonstrated that palm tocotrienol provided cardioprotection as evidenced by reduction of ischemia/reperfusion-mediated increases in ventricular dysfunction, ventricular arrhythmias and myocardial infarct size. Furthermore, Bilgin-Karabulut et al. [19] showed that pretreatment with a combination of Vitamins A and E gave protection against venous ischemia/reperfusion-induced injury. Interestingly, these vitamins were not effective when used as single agents.

In response to ischemia, cells activate various signal transduction pathways, which may be either harmful or allow adaptation to this stressful environment. Recent studies suggested that the MAPKs are important regulators of apoptosis in response to myocardial ischemia/reperfusion. Therefore, we characterized the three major MAPK subfamily members that are activated during ischemia and

reperfusion in our model and investigated the influence of dietary RPO on their phosphorylation status. Dietary RPO supplementation significantly increased p38 phosphorylation during reperfusion (Fig. 2A). Despite reports to the contrary [20,21], several investigators support the concept that p38 activation protects the heart from ischemia/reperfusion-induced injury [22,23]. These opposing results may be attributed to the different isoforms (α and β) expressed in the heart [24], which appear to mediate opposing effects. The p38α-isoform is implicated in apoptosis, whereas p38β is antiapoptotic in rat cardiac myocytes. JNK phosphorylation (JNK54 and JNK46) was significantly increased during reperfusion but was attenuated by dietary RPO supplementation. JNK phosphorylation appears to be proapoptotic in many cell types [25,26]; however, their exact role in regulating cell death is unclear. For example, Hreniuk et al. [27] found that inhibition of JNK46, but not JNK54, significantly reduced reoxygenation-induced apoptosis. Wang et al. [28], on the other hand, reported that activation of JNK by transfection of cultured rat neonatal cardiomyocytes with mitogen-activated protein kinase kinase 7 (MKK7), an upstream activator of JNK, induced hypertrophy rather than apoptosis. Although dietary RPO supplementation had no effect on ERK phosphorylation compared with the control group, the ERK cascade appears to specifically mediate cell growth and survival signals. For instance, it has been shown that inhibition of ERK enhances ischemia/reperfusion-induced apoptosis and that sustained activation of this kinase during simulated ischemia mediates adaptive cytoprotection in cultured neonatal cardiomyocytes [29].

We also investigated the involvement of PKB/Akt in the cellular response to dietary RPO supplementation. Red palm oil was responsible for a significant increase in PKB phosphorylation during reperfusion (Fig. 3). This is in agreement with the results of Fujio et al. [30] who showed that PKB/Akt promotes survival of cardiomyocytes in vitro and protects against ischemia/reperfusion injury in the mouse heart. PKB is activated downstream of PI3-K by the phosphoinositide-dependent protein kinases PDK-1 and PDK-2 [31]. PKB in turn phosphorylates a number of downstream targets relevant to cell survival functions, including the proapoptotic Bcl-2 family member BAD [32]. Phosphorylation of BAD on $Ser^{136}$ by PKB inhibits its proapoptotic function, thus promoting cell survival [33]. Interestingly, BAD is not only a substrate for PKB but is also phosphorylated by the MAPK kinase MEK [29], linking the classical Ras-MAPK pathway to cell survival.

Apoptosis has been consistently observed in cardiac myocytes after reperfusion and may represent a direct mechanism by which myocytes are damaged [34]. Indeed, in our model, reperfusion injury also resulted in cleavage of PARP to its proteolyzed products, a phenomenon well known to result from caspase-3 activation. Red palm oil supplementation significantly reduced PARP cleavage during reperfusion and attenuated caspase-3 activation, although not significantly.

In summary, our results have shown that dietary RPO supplementation caused increased phosphorylation of p38 and PKB, and reduce phosphorylation of JNK. Both increased PKB and p38 phosphorylation, and the inhibition of JNK phosphorylation may contribute to the protection of the cell against apoptosis. The attenuation of PARP cleavage would in turn be expected to inhibit apoptosis. Results presented in other studies [4,29], as well as our own [35], indicate that the MAPKs are central regulators of reactive signaling in cardiac myocytes. The ability to directly manipulate MAPK signaling has been shown to protect cardiomyocytes from ischemia/reperfusion-induced apoptosis/injury. This notion suggests that members of the MAPK signaling cascade would be ideal targets for pharmacological intervention to treat ischemia/reperfusion injury. Therefore, according to our results, a daily dosage RPO of 0.58mg/kg is recommended as being beneficial to humans.

In the current study, we have demonstrated for the first time that RPO might exert its beneficial effects during reperfusion through increased PKB/Akt and p38 phosphorylation and dephosphorylation of JNK, which might be associated with inhibition of apoptosis and improved function. Thus, RPO might offer an alternative nonpharmacological strategy to protect the heart against ischemia/reperfusion-induced injury.

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