Biochemical and Biophysical Research Communications 465 (2015) 719–724

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Role of melatonin, melatonin receptors and STAT3 in the cardioprotective effect of chronic and moderate consumption of red wine

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ARTICLE INFO

Article history:
Received 3 August 2015
Accepted 14 August 2015
Available online 18 August 2015

Keywords:
Red wine
Melatonin
Ischemia-reperfusion
Cardioprotection
SAFE pathway

ABSTRACT

We have recently discovered that melatonin, given acutely and directly to the isolated heart at the concentration found in wine, confers cardioprotection against ischemia-reperfusion (I/R). However, whether the presence of melatonin in wine contributes to the cardioprotective effect of chronic and moderate consumption of wine and its signalling mechanisms of protection are unknown. We therefore used both *in vivo* and *in vitro* models of I/R to investigate whether the presence of melatonin in red wine may contribute to the cardioprotective effect of chronic and moderate consumption of red wine. Wistar rats and C57black6 mice (WT) received drinking water supplemented daily with a moderate amount of red wine or melatonin given at the concentration found in the red wine. Rats were also pretreated with luzindole, a specific inhibitor of melatonin receptors 1 and 2 (2.3 mg/kg/day, intraperitoneally) or prazosin, a specific inhibitor of melatonin receptor type 3 (2.5 mg/kg/day, intraperitoneally). After 14 days, hearts were subjected to I/R *in vivo* or *ex vivo*. Red wine reduced the infarct size in both rats and WT mice ($p < 0.001$). Luzindole did not affect wine-induced cardioprotection, while prazosin reduced the infarct sparing effect of red wine ($p < 0.05$). Furthermore, red wine or melatonin failed to protect tumor necrosis factor alpha (TNF) receptor 2 knockout or cardiomyocyte specific signal transducer and activator of transcription 3 (STAT3) deficient mice (n.s. vs. control). Our novel findings suggest that the presence of melatonin in red wine contributes to the cardioprotective effect of chronic and moderate consumption of red wine against lethal I/R injuries. This effect is most likely mediated, at least in part, via melatonin receptor 3 and the activation of TNF and STAT3, both key players of the prosurvival and well described SAFE pathway.

1. Introduction

Chronic moderate consumption of red wine (2–3 glasses/day) is associated with a lower incidence of cardiovascular disease (CVD) (see review [1]). This phenomenon has been largely attributed to the alcohol content and the presence of polyphenols (mainly resveratrol) in wine. However, dealcoholized red wine still preserves cardiovascular benefits [2,3]. Most of the reported cardioprotective effects of polyphenols are mostly for concentrations higher than the plasma concentration obtained with daily moderate consumption of red wine (see review [1]). A recent prospective cohort study suggests that resveratrol levels achieved with diet does not have a substantial influence on health status (including cardiovascular disease) and mortality in humans [4], therefore suggesting that other cardioprotective components are present in wine. Delineating the exact cardioprotective components in red wine may provide a safe and inexpensive therapy against heart disease, without having the possible side effects of alcohol consumption.

Melatonin is a hormone well known for its powerful antioxidant properties [5] and its regulatory function on the circadian rhythm. Levels of melatonin are reduced in patients with acute coronary syndrome or after myocardial infarction (MI) (see review [6]), supporting experimental data demonstrating that melatonin confers cardioprotection against ischemia-reperfusion injury [7,8].

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http://dx.doi.org/10.1016/j.bbrc.2015.08.064
0006-291X/

Melatonin was recently discovered in food and the concentration of melatonin in red and white wine ranges from 5 to 300 pg/ml, a concentration range similar to human plasma concentrations (10–200 pg/ml) [9]. *In vitro*, an acute perfusion of melatonin at the concentration found in wine to an isolated mouse heart model, is sufficient to confer cardioprotection against MI [10]. However, whether oral administration of such a low dose of melatonin given chronically and most importantly, whether the presence of melatonin in red wine contributes to the cardioprotective effect of chronic and moderate consumption of red wine is unknown and is therefore the subject of the present study.

We used both *ex vivo* and *in vivo* models of MI in rats/mice to explore the role of melatonin in the cardiovascular benefits associated with daily moderate consumption of red wine for 2 weeks. With the use of pharmacological inhibitors of melatonin receptors and genetically modified animals, we explored how melatonin, its receptors and the Survivor Activator Factor Enhancement (SAFE) pathway (a key pro-survival pathway involving the activation of tumor necrosis factor alpha (TNF) and signal transducer and activator of transcription 3 (STAT3) as key components [11]), may contribute to the cardiovascular benefit of chronic consumption of red wine.

2. Materials and methods

2.1. Animals

All of the experiments conducted in male rats and mice were performed in accordance with the Guide for Care and Use of Laboratory animals published by the U.S. National Institutes of Health (NIH publication No. 85(23), revised 1996). Food and beverage were given ad libitum. Male Wistar rats (initial weight of rats, 200 g), male Tumour Necrosis Factor Receptor 2 knockout mice (TNFR2−/−) and their wild type controls (TNF-WT), cardiomyocyte-specific Signal Transducer Activator of Transcription 3-deficient mice (STAT3−/−) and their wild type littermate controls (12 weeks old) used in the study were bred in our Animal Unit as previously described [12]. All procedures were approved by the Animal Research Ethics Committee, University of Cape Town, Cape Town, South Africa.

2.2. Experimental protocol

The drinking water of control mice/rats was supplemented with a French cabernet sauvignon containing approximately 75 pg/ml of melatonin (which was diluted 1 part of wine into 7 parts of water), as previously described [3] or with melatonin (solution of 75 pg/ml diluted 1 part of melatonin solution into 7 parts of water). Prazosin (2.3 mg/kg/day, intraperitoneally), a melatonin receptor 3 inhibitor, luzindole (1 mg/kg/day, intraperitoneally), a melatonin receptor 1 & 2 inhibitor or AG490, (10 mg/kg, i.p.), an inhibitor of the STAT3 pathway, were given together or in the absence of red wine/melatonin treatment for 14 days prior to MI.

2.3. In vitro rat heart experiments

After 14 days of pretreatment with drinking water only, wine or melatonin, Wistar rats were anesthetized with sodium pentobarbitone (60 mg/kg, i.p.), hearts were excised rapidly and perfused retrogradely using the Langendorff perfusion technique as previously described [13].

All rat hearts underwent 30 min of stabilisation followed by 30 min of global ischaemia (at 37 °C) and 60 min of reperfusion. Both the functional parameters measured throughout the protocol and the infarct size assessed by 2,3,5 triphenyltetrazolium chloride (TTC) staining were determined as previously described [13].

2.4. Ex vivo mouse heart experiments

After 14 days of pretreatment with drinking water only, wine or melatonin, mice were anaesthetized with sodium pentobarbitone (60 mg/kg, intraperitoneally) and hearts were mounted on a Langendorff system as previously described [12]. After a 30 min stabilization period, hearts were subjected to 35 min of global ischemia followed by 45 min of reperfusion. Thereafter, infarct size (the ratio of dead tissue/healthy tissue of the entire heart) was assessed by TTC staining and infarct size was determined as previously described [12].

2.5. In vivo mouse heart experiments

Mice (C57BL6) were pretreated for 14 days with drinking water, red wine or melatonin. Mice were anaesthetized with ketamine (0.06 mg/ml) and xylazine (2 mg/ml) at a final dose of 0.01 ml/g [14]. Mice were then intubated at 120 strokes/min and 220 μl stroke volume, using a Minivent respirator (Harvard, USA). Thereafter, a left anterior thoracotomy was performed and the left ascending coronary artery was ligated (8.0 ethilon, nylon suture black filament), approximately 2 mm below the tip of the left atrium. A snare was created to induce 30 min of ischemia and then released for 120 min of reperfusion. Evans blue was injected into the left ventricle to delineate dead tissue. The hearts were stored at −20 °C and later sectioned and stained as previously described [12].

2.6. Western blot analysis

Ventricular tissues from water or wine-pretreated rat hearts were excised after 7 min of reperfusion, freeze-clamped in liquid nitrogen, and stored at −80 °C until further analysis. Proteins were extracted, phosphorylated STAT3 (phospho-STAT3 Tyr 705) and total levels of STAT3 in both nuclear and cytosolic fractions were analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis as previously described [15].

2.7. Statistical analysis

Data are presented as mean ± SEM. Comparisons between multiple groups were performed by one-way ANOVA followed by the Dunnet's *post hoc* test (Graph Pad Instat). Data obtained in knockout animals were analysed using a two-way analysis of variance (Graph Pad Prism 3.0). A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Chronic and moderate consumption of red wine or melatonin confer in vivo cardioprotection

Infarct size (expressed as a percentage of the area at risk) in control mice subjected to an *in vivo* ischemia-reperfusion insult was 60.0 ± 2.3% (Fig. 1a). Moderate and chronic consumption of red wine for 14 days dramatically reduced the infarct size to 23.3 ± 1.8% ($p < 0.001$ vs. control). Similarly, melatonin, given at the concentration found in wine for 14 days, reduced the infarct size to the same extent as red wine ($p < 0.001$ vs. control).

These findings were confirmed in an *ex vivo* rat model of MI (Fig. 1b). Isolated rat hearts subjected to an ischaemia-reperfusion insult had an infarct size of 60.8 ± 2.4% and a rate pressure product (RPP) (calculated as the ratio of left ventricular developed

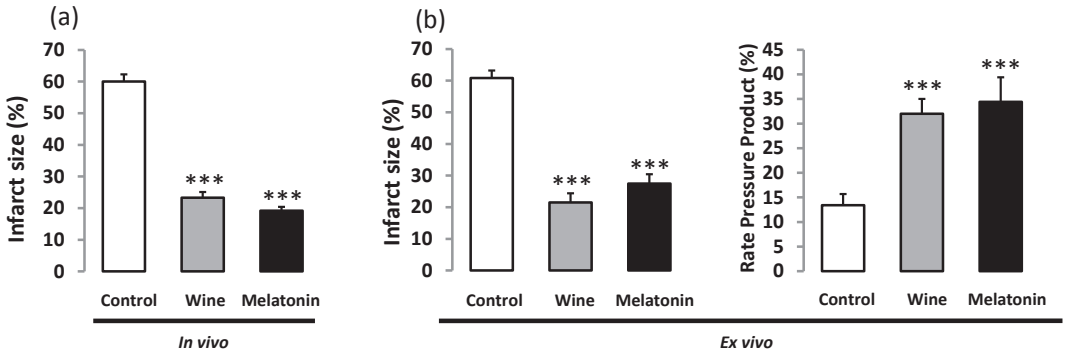

Fig. 1. Effect of daily moderate consumption of red wine or melatonin in (a) an *in vivo* mouse (C5BL6) heart model and (b) an *ex vivo* rat (Wistar) heart model of ischemia-reperfusion. n ≥ 5. ***p < 0.001 vs control group.

pressure multiplied by heart rate at baseline and at the end of the reperfusion period and expressed as a percentage of baseline value) of 13.4 ± 2.3%. Treatment with red wine for 14 days reduced the infarct to 21.5 ± 2.9% ($p < 0.001$ vs. control) and improved RPP to 32.0 ± 3.0% ($p < 0.01$ vs. control). Similarly, melatonin reduced infarct size to 27.5 ± 2.9% ($p < 0.001$ versus control) and improved RPP to 34.4 ± 5.0% ($p < 0.01$ vs. control). Of note, a chronic treatment with red wine or melatonin did not affect the body weights of the animals nor baseline hemodynamic data (data not shown).

3.2. Effect of melatonin receptor inhibitors on red wine-induced cardioprotection

To explore whether melatonin may contribute to red wine-induced cardioprotection, rats were treated for 14 days with either wine or melatonin in the presence of the melatonin inhibitors (luzindole or prazosin) prior to an I/R insult performed on the isolated rat heart perfusion model (Fig. 2). Chronic treatment with prazosin alone prior to the ischemic insult resulted in an infarct size of 57.6 ± 1.3% (n.s. vs. control). The protective effect of red wine was partially attenuated in the presence of prazosin ($p < 0.001$ vs. red wine). Similarly, the protective effect of melatonin was abolished in the presence of prazosin ($p < 0.001$ vs. melatonin).

Chronic treatment with luzindole alone did not affect the infarct size (ns vs. control). Luzindole blocked the infarct-sparing ability of melatonin (n.s. vs. control) but failed to reduce the cardioprotective effect of red wine (19.4 ± 0.7% n.s. vs. wine). Of note, none of the treatments affected the body weights of the animals prior to I/R.

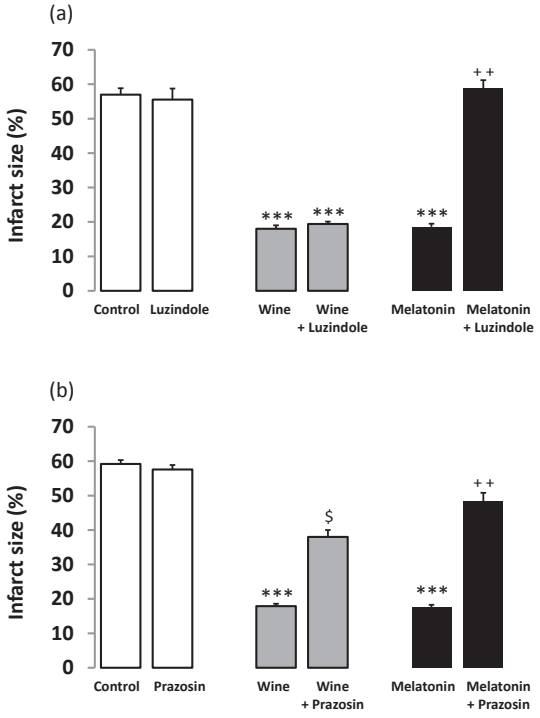

Fig. 2. Effect of melatonin receptor inhibitors on red wine and melatonin-induced cardioprotection. Infarct size was measured in isolated rat hearts subjected to an ischemia-reperfusion insult after 14 days of treatment with prazosin (an inhibitor of melatonin receptor 3)/luzindole (an inhibitor of melatonin receptors 1 and 2) and red wine or melatonin. n ≥ 5. ***p < 0.001 vs. control; ++p < 0.001 vs. melatonin; $p < 0.05 vs. wine.

3.3. Daily treatment with red wine or melatonin failed to confer cardioprotection in TNFR2−/− or STAT3−/− mice

Infarct size of isolated wild type control mouse hearts subjected to an *ex vivo* I/R insult was 52.9 ± 1.8% (Fig. 3a). Pretreatment with red wine or melatonin for 14 days reduced the infarct size in wild type hearts to 19.9 ± 1.5% and 15.3 ± 0.7%, respectively ($p < 0.001$ vs. control). In contrast, the cardioprotective effect observed with either red wine or melatonin was lost in TNFR2 deficient mice (Fig. 3a) or cardiomyocyte specific STAT3 deficient mice (Fig. 3b).

3.4. Chronic inhibition of STAT3 abolished the cardioprotective effect of daily moderate consumption of red wine

Isolated hearts from rats pretreated with AG490 had an infarct size and a RPP similar to the control group (n.s.) (Fig. 4a and b). However, this infarct-sparing effect was lost with AG490 (red wine + AG490: 57.9 ± 1.4%; n.s. vs. control) and reduced the functional recovery at the end of the reperfusion period (9.3 ± 1.8%, n.s. vs. control). Similarly, pretreatment with red wine for 14 days increased STAT3 phosphorylation in the nucleus compared to control hearts (*p < 0.05) (Fig. 4c).

4. Discussion

Our novel data strongly highlight, for the first time to our knowledge, that melatonin in red wine may play a major role in the cardioprotective effect afforded with chronic and moderate consumption of red wine. Chronic treatment of oral melatonin given at 75 pg/ml (a concentration similar to that present in red wine) protected the rat heart against I/R injury *in vivo* and *ex vivo*. The cardioprotective effect of red wine was partially abolished by prazosin, an inhibitor of melatonin receptor 3. Furthermore, a chronic treatment with melatonin or red wine failed to protect against I/R

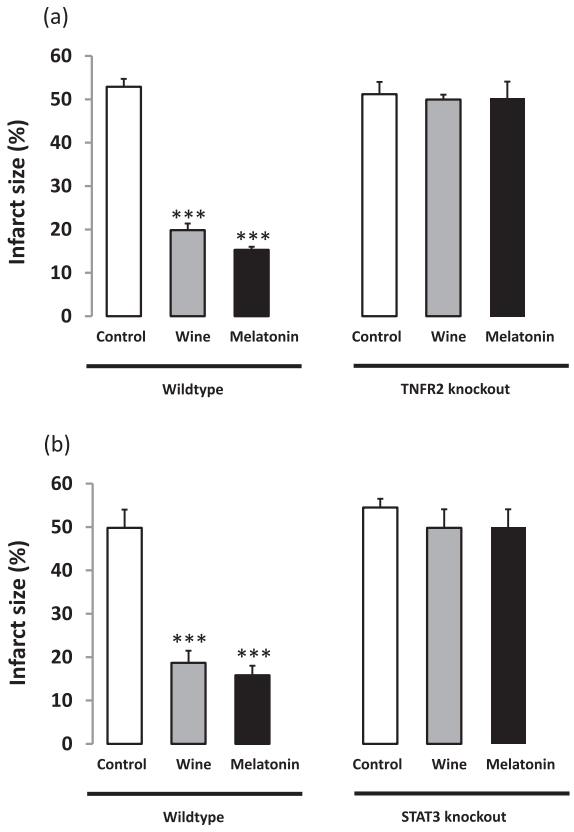

Fig. 3. Effect of chronic and moderate consumption of red wine or melatonin on infarct size in (a) TNF receptor 2-deficient mouse hearts or (b) cardiomyocyte specific STAT3 deficient mice hearts subjected to an *ex vivo* ischemia-reperfusion insult. n = 6. $p < 0.001$ vs. control.

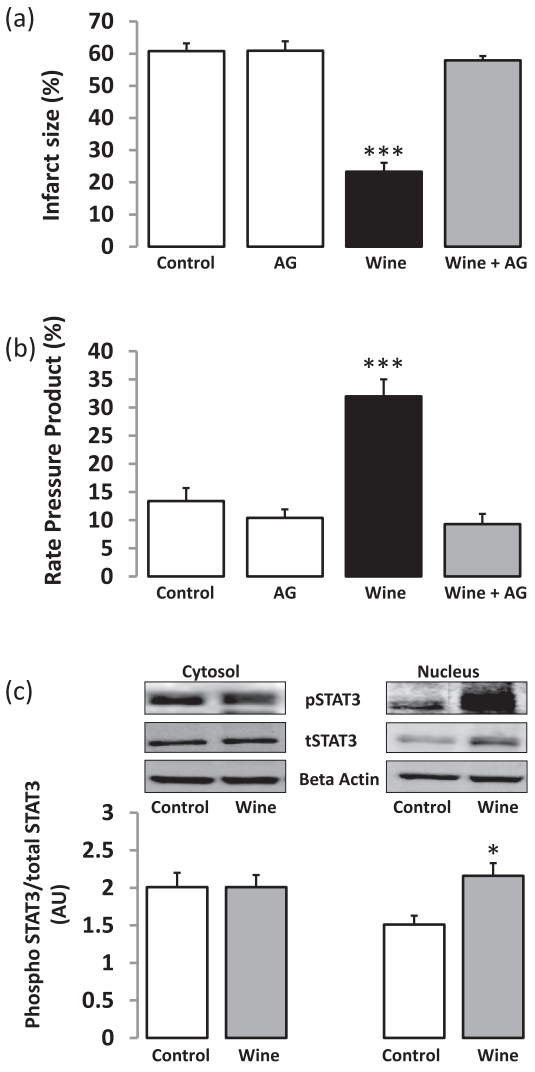

Fig. 4. Effect of AG490, an inhibitor of STAT3, on the cardioprotective effect of chronic and moderate consumption of red wine. (a) Infarct size and rate pressure product (b) (n = 6) were measured in isolated rat hearts subjected to an ischemia-reperfusion insult after 14 days of treatment with AG490 with/without wine. (c) Cardiac levels of phosphorylated STAT3/Total STAT3 in the cytosol and in the nucleus following a chronic treatment with wine and an ischemia-reperfusion insult (n = 3–4). *$p < 0.05$ ***$p < 0.001$ vs. control.

injury in TNF receptor 2 knockout or STAT3-deficient mice, therefore suggesting that red wine protects, at least in part, via the activation of melatonin receptor 3 and the SAFE pathway [11].

The French paradox, whereby the French present a relatively low mortality rate of coronary heart disease although they consume a diet rich in saturated fat, was attributed in 1992 to the daily and moderate consumption of red wine during the typical French meal [16]. Many epidemiological studies have since confirmed the cardiovascular benefits of red wine, but most of the experimental studies that have explored the cardioprotective effect of daily moderate consumption of red wine against acute myocardial infarction have given red wine extracts and not the wine itself (see review [1]). We have established an experimental model to demonstrate that daily moderate consumption of red wine improves cell death and functional cardiac parameters against I/R injury using the isolated rat heart Langendorff system [10]. Furthermore, our present data confirm the cardioprotective effect of daily moderate consumption of wine using an *in vivo* mouse model of acute myocardial infarction, which better mimics the clinical setting.

The prevalence of cardiovascular events and its resulting pathophysiological damage is directly related to the circadian rhythm. Myocardial infarct size and left ventricular function in reperfused MI patients have a circadian dependence [17]. Melatonin is a major regulator of the circadian rhythm [18]. Its concentration is reduced in patients with MI and low serum levels of melatonin inversely correlate with adverse post-infarct left ventricular remodelling [19].

Chronic administration of melatonin at a high concentration (40 μg/ml) protected the heart against I/R injury in an isolated working heart model [20]. Our previous data reported that acute administration of melatonin given to the heart directly, at the concentration found in red wine (75 pg/ml) protected the heart against I/R injury in an *in vitro* model [10] and against pulmonary hypertension in an *in vivo* model [21]. Both *in vitro* and *ex vivo*, our present data demonstrate that the concentration of melatonin found in 2–3 glasses of wine given daily for 14 days is sufficient to protect against acute myocardial infarction, to a similar extent as red wine given on its own. The content of melatonin in wine is much lower than the amount of melatonin prescribed for jet-lag (tablets of melatonin commercially available contain at least 1 mg of melatonin), but the consumption of one glass of beer or red wine in humans is enough to significantly increase serum melatonin content in a physiological range 1 h after its consumption (see review [22]). Melatonin is an important component in food included in the Mediterranean diet (i.e. olive oil, tomato, grapes) and as such, it may contribute to the cardiovascular benefit of this diet [22]. In humans, the health benefits of melatonin go beyond its

cardioprotective properties, including anti-diabetic effects [23], anti-cancer properties [24] and anti-depressive effects [18]. Whether these health benefits exist for the low amounts of melatonin achieved by nutrition (such as through regular consumption of red wine) remain to be investigated.

The cardioprotective effect of high doses of melatonin in animals have been attributed mainly via its binding to the melatonin receptors 1 (MT1) and 2 (MT2) [25]. We explored the effect of luzindole, an MT1 and MT2 antagonist, in red wine-induced protection. Luzindole inhibited the protective effect of melatonin, suggesting that melatonin on its own may protect via MT1 or MT2 receptors, but it failed to block the protective effect of red wine. It is therefore possible that the protective effect of a low concentration of melatonin in red wine is not mediated by these receptors and that the stimulation of these receptors may be dose-specific. Of important note, the pharmacodynamic of luzindole in conjunction with the various components in red wine is unknown, and its interaction with one of the 1000 components found in wine may have reduced the efficacy of the inhibitor.

Very little is known about melatonin receptor 3 (MT3) [26]. This receptor is not a typical membrane receptor but a very important enzyme denoted as the quinone reductase 2 (QR2) which plays an important role to balance the generation of free radicals in organisms. Melatonin can bind to this enzyme (MT3 binding site). It is believed that melatonin may function as a co-factor of this enzyme (see review [26]). To determine the role of the putative MT3 receptor, prazosin was administered in the presence or absence of red wine or melatonin. The infarct-sparing ability of melatonin was abolished in the presence of prazosin. Furthermore, the protective effect of red wine was diminished with the melatonin receptor 3 inhibitor. The fact that prazosin partially attenuated the infarct-sparing capacity of red wine suggests that a synergy of other compounds present in red wine may contribute to its cardioprotective effect. Of note, the effect of prazosin on melatonin's pathway may also be mediated via its modulation of adrenoreceptors [27]. The use of melatonin receptor-specific knockout mice would be useful to further characterize the exact role of each melatonin receptor in melatonin/red wine-induced cardioprotection.

The partial inhibition of red wine-induced cardioprotection in the presence of luzindole also suggests that melatonin is not the sole component in red wine to contribute to this effect. Quercetin, myricetin and other anthocyanins have been suggested to contribute to the cardioprotective effect of moderate and chronic consumption of red wine. The delineation of the contribution of these components in this effect is unfortunately very difficult to establish in either experimental or clinical conditions.

Melatonin is a powerful anti-oxidant, up to ten times more effective than vitamin E [28,29]. The powerful cardioprotective effect of melatonin on arrhythmias and infarct size in the isolated heart, may be mediated by its anti-oxidant properties [30]. Melatonin can also exert its protective effect by targeting mitochondrial function survival, through the restoration of oxidative status [31] or inhibition of the mitochondrial permeability transition pore opening [32]. In cardiomyocytes, melatonin activates the pro-survival kinase Akt, an effect mediated via its receptors, MT1 and MT2 [25]. Our present data demonstrate for the first time that the moderate and chronic consumption of red wine or low doses of melatonin for 14 days requires the activation of the pro-survival SAFE pathway for cardioprotection. Hence, melatonin activates the transcription factor STAT3 following the binding of TNF onto its specific TNFR2 (for further details, see review [11]). The pro-survival mechanisms of STAT3 are still unclear but may target the inhibition of the mitochondrial permeability transition pore opening required for the rescue of the cells [33–36].

Our data provide strong evidence that daily moderate consumption of red wine can protect the heart against an experimental I/R injury. Most importantly, inhibition of melatonin receptors in red wine resulted in the attenuation of red wine-induced protection, thus suggesting that melatonin may act as a key player in red wine-induced cardioprotection. The cardioprotective effect of both melatonin and red wine are mediated, at least in part, via the activation of the melatonin receptor 3 and the prosurvival SAFE pathway. Our data provide new insights for the explanation of the French paradox and most importantly, demonstrate that the amount of melatonin ingested through nutrition (and in particular through the Mediterranean diet) may be sufficient to confer cardiovascular benefits. Melatonin should be considered as a safe therapy in preventing cardiovascular disease in low- and high-income settings as melatonin is an inexpensive compound that is widely available over the counter in many countries.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This study was supported by the University of Cape Town, MRC South Africa, NRF and Winetech. Dr Paul Thomas is from the Division of Kinesiology & Health, University of Wyoming, USA. He was involved in this study while on sabbatical at the Hatter Institute with the support of the MRC South Africa.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.08.064.

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