Is red wine a SAFE sip away from cardioprotection? Mechanisms involved in resveratrol- and melatonin-induced cardioprotection

Abstract: Epidemiological studies suggest that regular moderate consumption of red wine confers cardioprotection but the mechanisms involved in this effect remain unclear. Recent studies demonstrate the presence of melatonin in wine. We propose that melatonin, at a concentration found in red wine, confers cardioprotection against ischemia–reperfusion injury. Furthermore, we investigated whether both melatonin and resveratrol protect via the activation of the newly discovered survivor activating factor enhancement (SAFE) prosurvival signaling pathway that involves the activation of tumor necrosis factor alpha (TNFα) and the signal transducer and activator of transcription 3 (STAT3). Isolated perfused male mouse (wild type, TNFα receptor 2 knockout mice, and cardiomyocyte-specific STAT3-deficient mice) or rat hearts (Wistars) were subjected to ischemia–reperfusion. Resveratrol (2.3 mg/L) or melatonin (75 ng/L) was perfused for 15 min with a 10-min washout period prior to an ischemia–reperfusion insult. Infarct size was measured at the end of the protocol, and Western blot analysis was performed to evaluate STAT3 activation prior to the ischemic insult. Both resveratrol and melatonin, at concentrations found in red wine, significantly reduced infarct size compared with control hearts in wild-type mouse hearts (25 ± 3% and 25 ± 3% respectively versus control 69 ± 3%, P < 0.001) but failed to protect in TNF receptor 2 knockout or STAT3-deficient mice. Furthermore, perfusion with either melatonin or resveratrol increased STAT3 phosphorylation prior to ischemia by 79% and 50%, respectively (P < 0.001 versus control). Our data demonstrate that both melatonin and resveratrol, as found in red wine, protect the heart in an experimental model of myocardial infarction via the SAFE pathway.

Introduction

Epidemiological studies suggest that moderate consumption of wine reduces the risk of cardiovascular disease, and the red wine hypothesis proposes that red wine is more likely to confer cardiovascular benefits than white wine (see review [1]). In experimental conditions, resveratrol, a polyphenol found mainly in red wine, offers cardiovascular benefit and is a strong argument favouring this hypothesis. At concentrations varying from 0.5 to 13.5 mg/L in red wine, resveratrol confers anti-ischemic effects via its antioxidant properties and via the activation of prosurvival pathways, such as the PI3-kinase/Akt pathway [2, 3].

The recent discovery of melatonin in red wine further supports the red wine hypothesis. This natural compound, well known to regulate the circadian rhythm in mammals, is also synthesized in various plants including grapes [4, 5]. The presence of melatonin in red wine is found to be superior to its concentration in white wines and ranges from 50 to 200 ng/L [6–8]. Experimental data suggest that a higher concentration of melatonin (40 mg/L) confers anti-ischemic effects but the exact mechanism remains unclear [9–12]. Therefore, whether melatonin, at a concentration as low as the one found in red wine, can confer cardioprotection is unknown.

Recently, we have discovered the novel intrinsic prosurvival survivor activating factor enhancement (SAFE) pathway to protect against ischemia–reperfusion injury [13]. This pathway involves the activation of the cytokine tumor necrosis factor alpha (TNFα), its receptor 2 (TNFR2), and the transcription factor signal transducer and activator of transcription 3 (STAT3) [14, 15]. However, whether resveratrol and melatonin may protect the heart via the activation of this pathway is unknown.

In the present study, we propose that both melatonin and resveratrol, at the concentration commonly found in red wine (75 ng/L and 2.3 mg/L respectively), confer cardioprotection against ischemia–reperfusion injury. Furthermore, we used genetically modified animals (TNFα receptor 2-deficient mice or cardiomyocyte STAT3-deficient mice) to demonstrate that both melatonin and resveratrol, at the concentration found in red wine, protect via the activation of the SAFE pathway.

Key words: cardioprotection; ischemia–reperfusion; melatonin; Red wine; resveratrol

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Methods

Animals

All the experiments conducted on 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). All procedures were approved by the Animal Research Ethic Committee, University of Cape Town, Cape Town, South Africa.

Isolated mouse heart model

TNFR2 (TNFR2\(^{-/-}\)) and their littermate controls (TNF-WT), cardiomyocyte-specific STAT3-deficient mice (STAT3\(^{-/-}\)) and their littermate controls (STAT3-WT) were used [14]. All mice (12–16 wk of age) were anaesthetized (60 mg/kg intraperitoneal sodium pentobarbitone) and mounted on a Langendorff system. After a 30-min stabilization period, hearts were subjected to 35 min of global ischemia followed by 45 min of reperfusion. Hearts were pretreated with either 75 ng/L melatonin or 2.3 mg/L resveratrol for 15 min followed by a 10 min washout period before global ischemia. At the end of each experimental protocol, the infarct size (the dead tissue/healthy tissue of the entire heart) was assessed by 2,3,5 triphenyltetrazolium chloride staining and infarct size was determined by using computerized planimetry, as previously described [14].

Isolated rat heart model

Wistar rats (230–300 g) were anesthetized with 60 mg/kg intraperitoneal sodium pentobarbitone and were given an intravenous injection of 200 IU heparin. Hearts were excised rapidly and perfused retrogradely using the Langendorff perfusion technique at a constant pressure (100 cm) with oxygenated Krebs-Henseleit buffer [16]. A balloon was inserted through the left ventricle, and the left ventricular end diastolic pressure (LVEDP) was adjusted between 4 and 8 mmHg. Cardiac parameters were monitored continuously and included heart rate (HR), left ventricular developed pressure (LVDP; is the difference between left ventricular end systolic pressure and the LVEDP) and the coronary flow.

The perfusion protocol is shown in Fig. 1. All rat hearts were equilibrated for 30 min and consequently subjected to a standard 30 min of regional ischemia (RI) with a 3/0 silk suture placed around the left coronary artery to form a snare. After the occlusion, the heart was reperfused for 120 min. Melatonin (75 ng/L) or resveratrol (2.3 mg/L) was perfused for 15 min followed by a 10-min wash out period before regional ischemia. Additional two groups were perfused with AG490 (100 nm, an inhibitor of the STAT3 pathway). AG490 was perfused for 3 min on its own followed by a 15-min co-administration with melatonin or resveratrol followed by a further 5 min of AG490 alone.

Western blot analysis

Ventricular tissues from control, melatonin, or resveratrol pretreated rat hearts were excised prior to the ischemic insult, freeze clamped in liquid nitrogen, and stored at \(-80^\circ\text{C}\). Phosphorylated states of STAT3 (phospho-STAT3 Tyr 705) and total levels of STAT3 were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis with antibodies from cell signaling technology, as previously described [17]. Equal loading was verified with Ponceau staining, and levels of phosphorylated proteins were normalized to their total protein levels performed in the same samples and under the same conditions but on a separate membrane. Relative densitometry was determined with use of a computerized software package, UVIBAND.

![Fig. 1. Experimental protocol for isolated rat heart perfusions. Melatonin (75 ng/L), resveratrol (2.3 mg/L) were given with/without the STAT3 inhibitor AG 490 (100 nm). S, stabilization; RI, regional ischemia; R, reperfusion; WB, Western blot analysis; D, drug (melatonin or resveratrol); A, AG 490; wo, washout.](image-url)
Statistical analysis

Data are presented as mean ± S.E.M. N = 6 per group. Comparisons between multiple groups were performed by one-way ANOVA followed by the Dunnett’s post hoc test (Graph Pad Instat, Graph Pad Software Inc, La Jolla, CA, USA). A value of $P < 0.05$ was considered as statistically significant.

Results

Melatonin confers cardioprotection but fails to protect TNFR2$^{-/-}$ and STAT3$^{-/-}$ mice

Isolated TNFR2 wild-type mouse hearts subjected to 35 min of global ischemia followed by 45 min of reperfusion presented an infarct of 69 ± 3% of the total heart (Fig. 2A). Pretreatment with melatonin (75 ng/L) reduced the infarct to 25 ± 2% ($P < 0.001$ versus control). Interestingly, melatonin failed to protect the heart in TNFR2$^{-/-}$ mice (62 ± 3%, nonsignificant (n.s) versus control group). Melatonin successfully reduced the infarct size in isolated STAT3 wild-type mouse hearts (52 ± 3 versus 20 ± 2, $P < 0.001$) but failed to reduce the infarct size in STAT3$^{-/-}$ mice (60 ± 3, ns versus respective control) (Fig. 2B).

Melatonin-induced cardioprotection requires STAT3 phosphorylation in isolated rat hearts

Isolated rat hearts subjected to regional ischemia–reperfusion had an infarct of 44 ± 3% (Fig. 3A). Pretreatment with melatonin did not alter the functional parameters of the heart (Table 1), but it reduced the infarct size to 25 ± 3% ($P < 0.001$ versus control group). However, co-administration of AG490, the STAT3 inhibitor, with

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Fig. 2. Melatonin protects against ischemia–reperfusion via the survivor activating factor enhancement (SAFE) pathway. Pretreatment of melatonin (75 ng/L) prior to an ischemia–reperfusion insult in isolated mice hearts reduces infarct size in wild-type animals but fails to protect TNF receptor 2 (TNFR2$^{-/-}$) mice (A) and cardiac STAT3-deficient mice (B). CTL, ischemic control; Mel, melatonin; WT, wild type; ***$P < 0.001$ versus IC in wild-type animals.

Fig. 3 Role of STAT3 in melatonin-induced cardioprotection

The infarct sparing observed with the pretreatment of melatonin (75 ng/L) prior to an ischemia–reperfusion insult in isolated rat hearts was lost in the presence of AG490, the STAT3 pathway inhibitor (A). Melatonin increases nuclear STAT3 phosphorylation prior to the ischemic insult (B). CTL, ischemic control; Mel, melatonin; A.U., arbitrary unit. ***$P < 0.001$, **$P < 0.01$ versus control group.
melatonin abolished the protective effect of melatonin (38 ± 9%, ns versus control group) (Fig. 3A). Pretreatment of the isolated heart with melatonin was also associated with an increased phosphorylation of STAT3 in the nucleus prior to the ischemic insult (Fig. 3B).

**Resveratrol confers cardioprotection but fails to protect TNFR2−/− and STAT3−/− mice**

Pretreatment with resveratrol in TNFR2 wild-type mice subjected to an ischemia–reperfusion insult reduced the infarct from 69 ± 3% to 25 ± 3% (P < 0.001) (Fig. 4A). However, resveratrol failed to protect the heart in TNFR2−/−-deficient mice (70 ± 2 versus 65 ± 2%, ns versus respective control group).

Similarly, resveratrol failed to improve the infarct size in STAT3-deficient mice while protection could be achieved in their littermate controls (Fig. 4B).

**Resveratrol-induced cardioprotection requires STAT3 phosphorylation in isolated rat hearts**

Pretreatment with resveratrol in isolated rat hearts subjected to an ischemia–reperfusion insult did not affect the functional parameters of the heart (Table 2) but it reduced the infarct to 15 ± 1% (P < 0.001 versus control group) (Fig. 5A). However, the addition of AG490, the STAT3 inhibitor, with resveratrol abolished the infarct sparing effect of resveratrol (50 ± 4%, ns versus control group).

Furthermore, pretreatment of resveratrol was associated with an increased phosphorylation of STAT3 in both the cytosol and the nucleus prior to the ischemic insult (Fig. 5B).

**Discussion**

This study demonstrates that a very low concentration of melatonin (corresponding to the concentration found in red wine) protects the heart against an ischemia–reperfusion insult. Furthermore, we demonstrate that both melatonin and resveratrol confer protection via the activation of the recently discovered SAFE pathway that involves the activation of TNFR2 and STAT3. Hence, both resveratrol and melatonin failed to protect against ischemia–reperfusion in hearts of TNFR2-deficient mice or cardiomyocyte STAT3-deficient mice. Similarly, the protection with resveratrol or melatonin in rat hearts was abrogated in the presence of AG490, an inhibitor of the STAT3 pathway. Furthermore, both resveratrol and melatonin pretreatment were associated with an increased activation of STAT3 in the nucleus, therefore suggesting an activation of STAT3 in the myocardium of treated rats.

**Melatonin, given at the concentration found in red wine, can confer cardioprotection**

The cardioprotective effect of melatonin against ischemia–reperfusion was previously reported both in vitro and in vivo at concentrations ranging from 1 to 50 μM (0.22–11 mg/L) [9, 12, 18]. These concentrations are up to a 1000 times higher than the physiological mammalian blood levels of melatonin, which will generally oscillate between 10 ng/L during the day and 200 ng/L at night [19]. The novelty of our work was to demonstrate that a physiological concentration of melatonin (75 ng/L), corresponding to the concentration found in natural food products such as red wine, can also protect the heart against an ischemia–reperfusion insult. Our data support previous work demonstrating that endogenous melatonin contributes to cardioprotection, with infarct size measured in pinealectomized rats subjected to ischemia–reperfusion being increased compared to normal rats [12, 20]. Melatonin is commercially available and prescribed to regulate the sleeping pattern at a dose ranging between 1 and 3 mg per day. This dose of melatonin would require drinking...
more than 1000 L of wine per day! We acknowledge the fact that the amount of melatonin given directly to the isolated heart is higher than the amount that the heart would receive after ingestion of red wine, but half an hour after drinking 100 mL of red wine, the amount of melatonin in the blood is increased by approximately 20% [6].

Our data demonstrate that melatonin confers a comparable protective effect to resveratrol but at a concentration 30,000 times lower. Considering the finding uncovered, melatonin may be considered as a primary antioxidant in red wine.

Melatonin protects via the activation of the SAFE pathway

As reactive oxygen species contribute to ischemia/reperfusion injuries, the cardioprotective effect of melatonin has been essentially attributed to its antioxidant capacities by scavenging reactive oxygen species (see review [21]), limiting mitochondrial electron leakage and inhibiting the opening of the mitochondrial permeability transition pore opening [11]. Melatonin can exert some cardioprotective properties independently of its antioxidant effect, by activation of the PI3/Akt pathway or inhibition of the pro-apoptotic p38 mitogen activated protein kinase [10]. Activation of the prosurvival intrinsic SAFE pathway is required for the protection of both ischemic pre- and postconditioning, two powerful phenomena to protect against ischemia–reperfusion injuries [14, 17, 22]. Multiple cardioprotective drugs, such as sphingosine-1 phosphate, ethanolamine, insulin, bradykinin or opioids, also protect via this pathway [23–26]. Using genetically modified animals, our data demonstrate that melatonin-induced cardioprotection requires the activation of two major components of the SAFE pathway, namely the receptor 2 of the cytokine TNFα and the transcription factor STAT3.

**Table 2. Role for STAT-3 in resveratrol-induced cardioprotection in the isolated rat heart subjected to ischemia–reperfusion**

<table>
<thead>
<tr>
<th>Hemodynamic parameters</th>
<th>Pre-ischemic</th>
<th>Ischemic</th>
<th>Reperfusion 5 min</th>
<th>Reperfusion 30 min</th>
<th>Reperfusion 60 min</th>
<th>Reperfusion 120 min</th>
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<tr>
<td>LVDP (mmHg)</td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>95.0 ± 4.4</td>
<td>52.4 ± 5.5</td>
<td>65.2 ± 6.8</td>
<td>62.2 ± 6.0</td>
<td>54.4 ± 4.4</td>
<td>44.8 ± 5.7</td>
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<td>Res</td>
<td>90.9 ± 3.0</td>
<td>41.7 ± 6.3</td>
<td>56.0 ± 6.4</td>
<td>45.7 ± 3.5</td>
<td>38.6 ± 4.3</td>
<td>35.1 ± 3.4</td>
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<tr>
<td>Res + AG490</td>
<td>89.0 ± 3.2</td>
<td>43.0 ± 4.6</td>
<td>67.3 ± 4.1</td>
<td>56.6 ± 6.6</td>
<td>53.3 ± 4.0</td>
<td>53.0 ± 8.0</td>
</tr>
<tr>
<td>AG490</td>
<td>92.6 ± 4.0</td>
<td>38.6 ± 2.6</td>
<td>53.1 ± 6.3</td>
<td>53.1 ± 6.3</td>
<td>46.3 ± 5.4</td>
<td>40.0 ± 3.8</td>
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<tr>
<td>Control</td>
<td>348 ± 6.1</td>
<td>308 ± 2.0</td>
<td>312 ± 10.0</td>
<td>308 ± 10.4</td>
<td>292 ± 14.7</td>
<td>292 ± 14.7</td>
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<tr>
<td>Res</td>
<td>314.0 ± 10.4</td>
<td>268.6 ± 11.4</td>
<td>285.7 ± 18.4</td>
<td>297.1 ± 4.8</td>
<td>297.1 ± 14.8</td>
<td>308.6 ± 14.2</td>
</tr>
<tr>
<td>Res + AG490</td>
<td>326.6 ± 16.0</td>
<td>333.3 ± 8.4</td>
<td>320.0 ± 20.7</td>
<td>313.0 ± 19.0</td>
<td>306.7 ± 22.3</td>
<td>367 ± 24.6</td>
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<td>AG490</td>
<td>320.0 ± 21.4</td>
<td>302.9 ± 24.5</td>
<td>274.3 ± 28.1</td>
<td>291.4 ± 24.2</td>
<td>302.9 ± 22.9</td>
<td>302.9 ± 19.2</td>
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<tr>
<td>Coronary flow (mL/min)</td>
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</tr>
<tr>
<td>Control</td>
<td>11.6 ± 0.6</td>
<td>7.0 ± 0.7</td>
<td>11.2 ± 0.7</td>
<td>12.4 ± 0.6</td>
<td>10.6 ± 0.9</td>
<td>10.0 ± 0.9</td>
</tr>
<tr>
<td>Res</td>
<td>11.4 ± 0.4</td>
<td>7.4 ± 0.8</td>
<td>11.4 ± 0.4</td>
<td>10.6 ± 0.7</td>
<td>10.6 ± 0.7</td>
<td>8.9 ± 0.9</td>
</tr>
<tr>
<td>Res + AG490</td>
<td>12.7 ± 1.2</td>
<td>8.3 ± 1.2</td>
<td>11.2 ± 1.0</td>
<td>11.0 ± 1.5</td>
<td>11.7 ± 1.2</td>
<td>8.0 ± 1.7</td>
</tr>
<tr>
<td>AG490</td>
<td>11.4 ± 0.6</td>
<td>8.0 ± 0.7</td>
<td>11.5 ± 0.4</td>
<td>11.1 ± 0.6</td>
<td>11.1 ± 0.6</td>
<td>9.4 ± 0.7</td>
</tr>
</tbody>
</table>

Parameters measured prior to ischemia (pre-ischemic), parameters measured after ischemia at 30 min of reperfusion (postischemic). Res, resveratrol; AG490, a STAT-3 inhibitor; Res + AG, co-administration of resveratrol and AG490; LVDP, left ventricular developed pressure; HR, heart rate; CF, coronary flow. All groups ns versus the control group at 120 min of reperfusion.

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**Fig. 4. resveratrol protects against ischemia–reperfusion via the survivor activating factor enhancement (SAFE) pathway.** Pretreatment of resveratrol (2.3 mg/L) prior to an ischemia–reperfusion insult in isolated mice hearts reduces infarct size in wild-type animals but fails to protect TNF receptor 2 (TNFR2−/−) (A) mice and cardiac signal transducer and activator of transcription 3 (STAT3)-deficient mice (B). CTL, ischemic control; RES, resveratrol; WT, wild type; ***p < 0.001 versus CTL in wild-type animals.
Resveratrol protects against ischemia reperfusion injury via the SAFE pathway

In patients with coronary artery disease, dealcoholized red wine (DRW) decreased arterial stiffness [27, 28] and in apolipoprotein E-deficient mice, DRW limits atherosclerosis, thus providing conclusive evidence that red wine properties ‘go beyond alcohol’ [29].

Our data confirm numerous previous reports demonstrating that a concentration of resveratrol as found in red wine protects against ischemia–reperfusion injury [30]. Again, a limitation of our study and others is the fact that the amount of resveratrol given directly to the isolated heart is higher than the amount that the heart would receive after ingestion of red wine. Following an acute oral administration of red wine in rats (4 mL of an Italian red wine containing 6.5 mg/L of resveratrol), the resveratrol concentration peaked at 20 μg/L in the plasma [31]. Although chronic red wine consumption may be associated with higher concentrations than those measured with an acute treatment, several studies have demonstrated that resveratrol as low as 0.22 mg/L can exert a number of biologic effects [31]. Additional in vivo and chronic animal studies are required to confirm that this effect can account for the cardioprotective effect of red wine.

The mechanisms of protection for resveratrol have been extensively studied but still remain uncertain. Well known for its antioxidant properties [32], the anti-ischemic effect of resveratrol is thought to be mediated via activation of nitric oxide, adenosine, Akt, Bcl-2 and inactivation of pro-apoptotic factors such as Bad and glycogen synthase kinase 3β [3, 31, 33]. Our data delineate a novel prosurvival pathway that resveratrol can activate to limit ischemia–reperfusion injury, and we suggest that resveratrol can target the immune system to limit ischemia–reperfusion damage by activating TNF-α.

As both resveratrol and melatonin, at concentrations as found in red wine confer cardioprotection by activation of the SAFE pathway, we suggest that moderate consumption of red wine protects against ischemic heart disease by activation of the SAFE pathway. However, additional experiments conducted in vivo with a chronic consumption of melatonin, resveratrol, or red wine will be required to confirm our statement as synergistic effects may occur between the different compounds in red wine.

Conclusion

In conclusion, our data strongly support the fact that the presence of melatonin in red wine, together with resveratrol, may contribute to the red wine hypothesis it also suggests that melatonin is a superior antioxidant present in red wine and contributes to the red wine hypothesis. Furthermore, we have delineated a novel mechanism by which low amounts of melatonin and resveratrol protect the heart via the activation of the powerful prosurvival SAFE pathway, which involves the activation of both TNF-α and STAT3. Our data provide exciting novel insight into the use of natural compounds in the treatment of cardiac disease.

Acknowledgements

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Conflict of interest
The authors declared no conflict of interest.

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23. FUGLESTEG BN, SULEMAN N, TIRON C et al. Signal transducer and activator of transcription 3 is involved in the cardioprotective signalling pathway activated by insulin therapy at reperfusion. Basic Res Cardiol 2008; 103:444–453.