Protective Antioxidant Effects of Carvedilol in a Rat Model of Ischaemia-reperfusion Injury

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This study investigated the protective effects of carvedilol, a potent antioxidant, in a rat model of tourniquet-induced ischaemia-reperfusion injury of the hind limb. Thirty rats were divided equally into three groups: the control group (group 1) was only anaesthetized, without creating an ischaemia-reperfusion injury; group 2 was submitted to ischaemia (4 h), followed by a 2-h reperfusion period; and group 3 was pre-treated with carvedilol (2 mg/kg per day) for 10 days prior to ischaemia-reperfusion. Ischaemia-reperfusion produced a significant decrease in superoxide dismutase and glutathione peroxidase activities in the liver, lungs, muscle and serum compared with control treatment, and pre-treatment with carvedilol prevented these changes. Ischaemia-reperfusion caused a significant increase in malondialdehyde and nitric oxide (NO) levels in liver, lungs, muscle (except NO) and serum compared with control treatment, and carvedilol prevented these changes. In conclusion, it might be inferred that carvedilol could be used safely to prevent oxidative injury during reperfusion following ischaemia in humans.

KEY WORDS: CARVEDILOL; ANTI-HYPERTENSIVE AGENT; ISCHAEMIA-REPERFUSION INJURY; REACTIVE OXYGEN SPECIES

Introduction

Ischaemia-reperfusion injury is a deleterious clinical condition that occurs when blood circulation is restored following an episode of acute tissue ischaemia. The interruption of the blood supply results in ischaemia, which rapidly damages metabolically active tissues. Paradoxically, the restoration of blood flow initiates a cascade of events that may lead to cell and tissue damage. The risk of mortality and morbidity from ischaemia-reperfusion injury still remains an important medical problem associated with several clinical situations, including acute peripheral arterial occlusion (emboli or thrombi), stroke, acute myocardial ischaemia, haemorrhagic shock and organ transplantation. The same consequences may be encountered in procedures such as coronary angioplasty, coronary artery bypass surgery or revascularization of an ischaemic extremity.1 Acute arterial occlusion still remains the most common disorder of all vascular diseases, accounting
for 10 – 16% of the reported cases in the literature.²

Ischaemia-reperfusion injury of skeletal muscle involves a series of deleterious events including free oxygen radical production, intracellular calcium overload, damage to the microvasculature, lipid peroxidation, and no-reflow phenomenon. Cell death is usually the final stage in this scenario.³

Calcium overload and oxidative stress are the two main hypotheses that have been proposed to explain the pathogenesis of ischaemia-reperfusion injury. Both of these mechanisms are related; however, it is not clear how they interact in the chain of damaging events.

Oxidative stress is associated with increased reactive oxygen species (ROS). ROS, particularly the hydroxyl radical, interact with phospholipids, proteins and nucleic acids leading to lipid peroxidation, which results in a loss of membrane integrity.⁴ Lipid peroxidation is the most important damaging effect of free radicals. Malondialdehyde (MDA) is a by-product of radical-induced lipid peroxidation, and it can be used as a marker of free radical formation.⁵ – ⁷ The tissue damage caused by the production of ROS can trigger several defence mechanisms. The first-line defence mechanism includes antioxidants such as superoxide dismutase (SOD), catalase and glutathione peroxidase (GSH). These enzymes catalyse the conversion of ROS into less reactive species.⁶,⁷ GSH and SOD are the most important endogenous antioxidant defence mechanisms against oxidative stress.

There are also some exogenous antioxidants that might help to improve cellular integrity in ischaemia-reperfusion injury. It has been suggested that drugs such as β-adrenergic blockers, angiotensin-converting enzyme inhibitors and calcium-channel blockers are exogenous antioxidants.⁸

Carvedilol is a vasodilating antihypertensive drug that selectively blocks α₁-receptors and non-selectively antagonizes β₁- and β₂-adrenoceptors. Carvedilol and some of its metabolites are also potent antioxidants.⁸ In this study, the antioxidant effect of carvedilol was assessed in the rat skeletal muscle ischaemia-reperfusion injury model.

Materials and methods

STUDY GROUPS

The study was conducted on 30 male adult Sprague–Dawley rats weighing 250 – 350 g. The protocol for this study was approved by the Ethics Committee of Kocaeli University School of Medicine, which follows the National Institutes of Health Guidelines of Care and Use of Experimental Animals.⁹

Each study group consisted of 10 rats: group 1 (control group) rats were anaesthetized with ketamine only; group 2 (ischaemia-treated group) rats were reperfused for 2 h following a 4-h ischaemic period; and group 3 (carvedilol-treated group) animals were pre-treated with 2 mg/kg per day of oral carvedilol (Dilatrend®, 6.25 mg, Roche, Istanbul, Turkey) for 10 days via a nasogastric tube prior to ischaemia-reperfusion. The oral dosage chosen followed that established by Necas et al.¹⁰

RAT SKELETAL MUSCLE ISCHAEMIA AND REPERFUSION MODEL

Latex tourniquets were applied to the root of the hind limb of the rats as proposed by Strock and Majno.¹¹ Total ischaemia was confirmed by the absence of an arterial pulse distal to the tourniquet with the aid of Doppler ultrasonography (MD2, Huntleigh Diagnostics Ltd, South Glamorgan, UK).
After a 4-h ischaemic period, the tourniquet was removed and the limb was reperfused for 2 h. Reperfusion was confirmed by the reappearance of an arterial pulse in the limb circulation by Doppler ultrasound. All of the rats were anaesthetized with ketamine hydrochloride 100 mg/kg intraperitoneally (500 mg flacon, Ketalar®, Pfizer, Istanbul, Turkey) and sacrificed. Tissue biopsies were obtained from the gastrocnemius muscle, liver and lungs. Blood samples were drawn from the aorta and heart by puncture. MDA, GSH, SOD and nitric oxide (NO) levels were determined in tissue biopsies and blood samples.

**BIOCHEMICAL ASSAYS**

The tissues were removed and washed three times in cold isotonic saline (0.9% [v/w]). Tissues were homogenized with cold Tris/HCl buffer (pH 7.4) to make a 10% homogenate (w/v). Tissue lipid peroxide levels, expressed in terms of MDA levels – end product of lipid – were determined according to the method of Buege and Aust.\(^\text{12}\) The results were expressed as nmol/100 mg protein in tissue and as µmol/l in serum. Tissue GSH was measured according to the method of Ellman with 5,5'-dithiobis-(2-nitrobenzoate).\(^\text{13}\) Homogenate (1 ml) was added to 1 ml of 10% trichloroacetic acid. After centrifugation, 4.5 ml of Ellman reagent in phosphate buffer (0.1 M, pH 8.0) was added to 0.5 ml of supernatant, and the absorbance was measured at 412 nm. The results were expressed as nmol/100 mg protein in tissue and mg/100 ml in serum samples.\(^\text{13}\) The protein concentrations of tissue homogenates were determined by the Lowry protein assay.\(^\text{14}\) The Cu,ZnSOD activity was measured kinetically by a method described by Sun et al.\(^\text{15}\) The principle of the method is based on the inhibition of nitroblue tetrazolium reduction by the xanthine–xanthine oxidase system as a superoxide generator. SOD activity was expressed as units/ml for serum and units/mg of tissue protein. The tissue NO measurements were based on the Griess reaction.\(^\text{16}\) It is very difficult to measure NO in biological samples, so tissue nitrate (NO\(_3^-\)) and nitrate (NO\(_2^-\)) levels were estimated as an index of NO production. Samples were initially deproteinized with Somogyi reagent.\(^\text{17}\) Total nitrate (nitrite + nitrate) was measured after conversion of nitrate into nitrite by copperized cadmium granules by a spectrophotometer at 545 nm. A standard curve was established with serial dilutions (10\(^{-8}\) – 10\(^{-3}\)) of sodium nitrite. Results were expressed as µmol/g of protein in tissues and µmol/l in serum. The blood samples were centrifuged in potassium ethylene diaminetetra-acetic acid (EDTA) containing tubes at 1000 \(\text{g}\) for 10 min at 4 ºC to remove the plasma. The preparation procedure for each sample was performed at 4 ºC.

**STATISTICAL ANALYSIS**

Values are presented as mean ± SD. Statistical analysis was performed using the one-way analysis of variance (ANOVA) test, followed by the Dunnett’s test, which was applied to calculate the statistical significance between various groups. \(P\)-values of < 0.05 were considered to be statistically significant. Statistical analyses were performed using the SPSS® version 10.0 software package (Chicago, IL, USA).

**Results**

**EFFECT OF CARVEDIOL PRE-TREATMENT ON LIPID PEROXIDATION**

Ischaemia-reperfusion injury to the muscle caused a significant increase in MDA levels in liver \((P = 0.005)\), lungs \((P = 0.015)\) and gastrocnemius muscle tissues \((P = 0.003)\).
H Akbas, M Ozden, M Kanko et al.

Antioxidant effects of carvedilol

Compared with the control group (Table 1). Carvedilol pre-treatment (2 mg/kg) prior to ischaemia-reperfusion reduced MDA levels in tissue samples, and there were no significant differences in tissue MDA levels between groups 1 (control) and 3 (carvedilol-treated) (Table 1).

Ischaemia-reperfusion injury to the muscle caused a significant increase in MDA levels in serum samples compared with the control group ($P < 0.001$, Table 2). Carvedilol pre-treatment prior to ischaemia-reperfusion reduced MDA levels in serum samples, and there were no significant differences in

<table>
<thead>
<tr>
<th>TABLE 1: Tissue lipid peroxidation, antioxidant activity and nitric oxide measurements in control rats and in rats exposed to ischaemia-reperfusion injury with and without carvedilol pre-treatment ($n = 30$)</th>
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<tr>
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<tr>
<td>Liver MDA (nmol/100 mg protein)</td>
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<td>Lung MDA (nmol/100 mg protein)</td>
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<td>Muscle MDA (nmol/100 mg protein)</td>
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<td>Liver SOD (U/mg protein)</td>
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<td>Lung SOD (U/mg protein)</td>
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<td>Liver GSH (nmol/100 mg protein)</td>
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<td>Liver NO (µmol/g protein)</td>
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<td>Muscle NO (µmol/g protein)</td>
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</tbody>
</table>

Results are expressed as mean ± SD. One-way ANOVA followed by Dunnett’s test were used on the values ($P < 0.05$). MDA, malondialdehyde; SOD, superoxide dismutase; GSH, glutathione peroxidase; NO, nitric oxide; NS, not significant.
serum MDA levels between groups 1 (control) and 3 (carvedilol-treated) (Table 2).

**EFFECT OF CARVEDILOL PRE-TREATMENT ON ANTIOXIDANT ACTIVITY**

Ischaemia-reperfusion injury to the muscle significantly decreased SOD activity in liver, lungs and muscle tissues compared with the control group (P = 0.049, P = 0.023, and P = 0.017, respectively; Table 1). In the ischaemia-reperfusion groups, tissue GSH levels demonstrated a significant decrease compared with the control group (P = 0.046, P = 0.005 and P = 0.020, respectively; Table 1). Carvedilol pre-treatment significantly prevented the ischaemia-reperfusion-induced reduction in tissue SOD and GSH levels, and there were no significant differences in the tissue SOD and GSH levels between groups 1 (control) and 3 (carvedilol-treated) (Table 1).

Ischaemia-reperfusion injury to the muscle caused a significant decrease in SOD and GSH levels in serum samples compared with the control group (P = 0.001 and P < 0.001, respectively; Table 2). SOD and GSH levels were significantly improved by carvedilol pre-treatment prior to ischaemia-reperfusion, and there were no significant differences in the serum SOD and GSH levels between groups 1 (control) and 3 (carvedilol-treated) (Table 2).

**EFFECTS OF CARVEDILOL PRE-TREATMENT ON NITRIC OXIDE LEVELS**

Ischaemia-reperfusion injury to the muscle caused a significant increase in NO levels in liver, lung and serum samples compared with the control group (P = 0.001, P = 0.001 and P = 0.011, respectively; Tables 1 and 2). The increase in NO level observed in the gastrocnemius muscle following ischaemia-reperfusion injury was not statistically significant (Table 1). The carvedilol-treated group did not show any significant differences in tissue and serum levels of NO compared with the control group (Tables 1 and 2).

**Discussion**

Acute arterial occlusion is known to be the most common clinical dilemma of all vascular disorders. It has been estimated that ischemia-reperfusion injury accounts for

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**TABLE 2**

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 10)</th>
<th>Ischaemia (n = 10)</th>
<th>Ischaemia–Carvedilol (n = 10)</th>
<th>Control–ischaemia P-values</th>
<th>Control–carvedilol P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (µmol/l)</td>
<td>12.81 ± 7.56</td>
<td>126.28 ± 84.00</td>
<td>22.06 ± 14.49</td>
<td>&lt; 0.001</td>
<td>NS</td>
</tr>
<tr>
<td>SOD (U/ml)</td>
<td>94.38 ± 16.98</td>
<td>58.92 ± 8.62</td>
<td>82.38 ± 7.92</td>
<td>0.001</td>
<td>NS</td>
</tr>
<tr>
<td>GSH (mg/100 ml)</td>
<td>52.89 ± 8.82</td>
<td>29.03 ± 7.67</td>
<td>54.91 ± 6.99</td>
<td>&lt; 0.001</td>
<td>NS</td>
</tr>
<tr>
<td>NO (µmol/l)</td>
<td>26.33 ± 3.04</td>
<td>39.68 ± 6.25</td>
<td>19.50 ± 12.46</td>
<td>0.011</td>
<td>NS</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD. One-way ANOVA followed by Dunnett’s test were used (P < 0.05) on the values. MDA, malondialdehyde; SOD, superoxide dismutase; GSH, glutathione peroxidase; NO, nitric oxide; NS, not significant.
Antioxidant effects of carvedilol

approximately 10 – 16% of all cases and this rate tends to get higher due to the increasing incidence of atherosclerotic vascular disease. This severe condition especially affects the older population, who have other coexisting problems such as hypertension, diabetes mellitus and coronary artery disease.

The rat skeletal muscle ischemia-reperfusion injury model simulates the same pathophysiological events of acute arterial occlusion. Cellular damage and probable death are the most common consequences of ischemia-reperfusion injury. Hypoxia, as a cause of prolonged ischemia, triggers anaerobic metabolism. As a result of depletion of intracellular adenosine triphosphate and raised lactate levels, deep acidosis accelerates the vicious cycle of events. The efflux of intracellular potassium-magnesium and the influx of extracellular calcium ions also stop vital cellular functions. Although restoration of the blood flow is critical for reversing ischemia, paradoxically the insult to the tissues worsens.

The production of excess ROS, especially by the xanthine oxidase system, and the participation of neutrophils increases the inflammatory reaction, thereby promoting muscular oedema formation, tissue necrosis, impairment of systemic clinical conditions and, sometimes, leading to limb loss and even death. After ischemia, tissues were shown to contain an accumulation of xanthine oxidase. This enzyme uses molecular oxygen, which is available as the final electron acceptor. This reaction produces singlet oxygen molecules, which are extremely unstable. Using these molecules, secondary chemical reactions produce superoxide, hydrogen peroxide and hydroxyl. The ROS start the lipid peroxidation, which ends in the disintegration of membranes and consequent rupture and death of the cell. The post-ischemic endothelium is the main source of the superoxide radical. Indirectly, superoxide may be responsible for the production of the damaging hydroxyl radical. Under normal conditions, the damaging effects of superoxide are prevented by SOD, which converts superoxide into hydrogen peroxide. However, during reperfusion of ischemic tissues, these natural defences may be overcome and hydrogen peroxide is converted into a hydroxyl radical, which in turn is capable of damaging a wide variety of biological molecules including amino acids, membrane transport proteins and nucleic acids. GSH is an important antioxidant, and the increased concentration of GSH in cells may be useful in the prevention of oxidative damage of endothelial cells. GSH is part of the defensive strategy that cells and tissues use to combat oxidation. Thus, SOD and GSH are the first-line enzymes that catalyse the conversion of ROS into less reactive forms.

The most important damaging effect of free radicals on tissues is lipid peroxidation. The cell membrane is composed of fatty acids and phospholipids. Oxygen free radicals cause cellular injury by inducing lipid peroxidation, which results in functional and structural cell alterations. Thus, MDA may indicate the presence of lipid peroxidation. The endothelium is also an important source of NO, a mediator of ischemia-reperfusion injuries. NO is synthesized from L-arginine by NO synthase and can act in a tissue-protective manner through physiological regulation of vascular tone, inhibition of platelet aggregation, attenuation of leucocyte adherence, scavenging oxygen-derived free radicals and maintenance of normal vascular permeability.
H Akbas, M Ozden, M Kanko et al.

Antioxidant effects of carvedilol

In this study we employed a previously studied model of tourniquet-induced skeletal muscle ischaemia-reperfusion injury.\textsuperscript{11} Tourniquet-induced skeletal muscle ischaemia-reperfusion injury is associated with free radical production and pro-inflammatory responses.\textsuperscript{21} Although pre-treatment with carvedilol improves the skeletal muscle ischaemia-reperfusion injury, the mechanism of this effect was unknown. The aim of this study was to assess the hypothesis that carvedilol attenuates skeletal muscle ischaemia-reperfusion injury.

Although it is known that carvedilol ameliorates cardiac, renal and neurogenic ischaemic tissue injury, its antioxidant effects on skeletal muscle ischaemia-reperfusion injury have not, to our knowledge, been reported previously in the literature.\textsuperscript{22–26} In our experimental study, the tourniquet-induced hind limb ischaemia-reperfusion injury caused a significant increase in MDA and NO levels and a significant decrease in the antioxidant enzyme activation of SOD and GSH in liver, lungs, muscle (except NO) and serum samples of rats. These findings demonstrated that carvedilol may attenuate the local and systemic damage that results from skeletal muscle ischaemia-reperfusion injury. The results also revealed that carvedilol pre-treatment prior to ischaemia-reperfusion injury can attenuate lipid peroxidation, as measured by MDA levels, at both the local (gastrocnemius muscle) and systemic (liver and lungs) tissue levels, and in serum samples; and that carvedilol can prevent the depletion of SOD and GSH levels caused by the ischaemia-reperfusion injury.

The increased SOD and GSH levels in tissue and serum samples may be due to the free radical scavenging and antioxidant effects of carvedilol. Our results also show that carvedilol pre-treatment significantly attenuated the NO increase in liver, lungs, and serum. The levels of NO in muscle tissue showed no significant change.

Carvedilol is a third-generation, non-selective $\beta_1$, $\beta_2$ and selective $\alpha_1$-blocker, which has potent antioxidant properties and is used in the treatment of hypertension, angina and congestive heart failure.\textsuperscript{26} Recently, novel therapeutic interventions have been developed that aim to improve muscle injury due to ischaemia-reperfusion.\textsuperscript{24,25} Carvedilol and its metabolites are potent antioxidants. The main metabolite of carvedilol, the hydroxylated compound SB 211475, has almost no $\beta$-receptor antagonist activity (170 times less active than carvedilol), but it has a greater antioxidant activity. This activity has been attributed to the carbozole moiety of carvedilol.\textsuperscript{5,24,26} It was reported recently that carvedilol has an endothelial-protecting function after ischaemia-reperfusion injury,\textsuperscript{27} and that it suppresses superoxide activity in neutrophils.\textsuperscript{28} Carvedilol preserves endogenous antioxidants such as GSH, which normally protect against oxidative stress.\textsuperscript{24,29} ROS can lead to structural changes in endothelial cells, but they can also indirectly affect endothelial function by reacting with the vasodilatory mediator NO, which readily reacts with superoxide to form peroxynitrite and peroxynitrous acid; this reaction can initiate lipid peroxidation.\textsuperscript{5} Carvedilol protects against peroxinitrite toxicity.\textsuperscript{24} The mechanism of action of the antioxidant effects of carvedilol is not fully understood, but it may be due to inhibition of lipid peroxidation by scavenging free radicals.\textsuperscript{30,31}

In conclusion, this study demonstrates that carvedilol can attenuate skeletal muscle ischaemia-reperfusion injury, probably with its antioxidant and endothelial-protecting
properties. It might therefore be inferred that this established anti-hypertensive drug could be used safely in humans to prevent oxidative injury during reperfusion following ischaemia.

Conflicts of interest

No conflicts of interest were declared in relation to this article.

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536