Carvacrol Protects against Acute Myocardial Infarction of Rats via Anti-oxidative and Anti-apoptotic Pathways

Wei Yu,* Qing Liu, and Sujuan Zhu

Department of Emergency, People’s Hospital of Zhangqiu City; Zhangqiu 250200, China.

Received October 28, 2012; accepted January 7, 2013; advance publication released online February 4, 2013

Carvacrol (CAR), a naturally occurring phenolic monoterpene, has been shown to possess diverse biological activities. The present study was undertaken to evaluate the cardioprotective potential of CAR against myocardial ischemic damage in a rat model of acute myocardial infarction. CAR significantly diminished the infarct size and myocardial enzymes including creatine kinase (CK), the MB isoenzyme of creatine kinase (CK-MB), lactate dehydrogenase (LDH) and cardiac troponin T (cTnT). Reduced level of malondialdehyde (MDA), obviously elevated activities of superoxide dismutase (SOD) and non-enzymatic scavenger glutathione (GSH) as well as glutathione peroxidase (GSH-PX) were also found in CAR-treated groups. Treatment with CAR remarkably inhibited the protein expressions of caspase-3 and Bax, but increased the level of Bcl-2 protein in infarcted rats by Western blot analysis. The finding suggests that the cardioprotection of CAR associate with its anti-oxidative and anti-apoptotic properties in acute myocardial infarction of rats.

Key words carvacrol; acute myocardial infarction; cardioprotection; anti-oxidative; anti-apoptotic

Cumulative evidence has demonstrated that reactive oxygen species (ROS) is one of the primary factors contributing to cell death in myocardial infarction. A previous investigation revealed that excessive formation of oxygen free radicals following ischemia not only destroyed cellular structures, but also caused mitochondrial dysfunction finally activating apoptotic signaling cascades. In addition, excessive free radicals was shown to attack the fatty acids within myocardial membranes and caused a chain reaction of lipid peroxidation, which had a detrimental effect on myocardium during acute myocardial infarction. Taken into account the notion that myocardial ischemia is linked with oxidative stress, it is of interest to investigate whether the cardioprotective drugs attenuate the damage of ROS due to ischemia.

Programmed cell death (apoptosis) is believed to be one of the major deleterious factors resulting in myocardium injury during myocardial infarction. Caspase-3, the major form of caspase, is served as an “apoptotic executor,” in response to the apoptotic stimuli. It was previously reported that a dramatic elevation of caspase-3 was observed in isoproterenol-induced acute myocardial infarction in Wistar rats myocytes. In addition to caspases, Bcl-2 family proteins have been also shown to play a pivotal role in the modulation of cellular apoptosis. Bcl-2 is a cytosolic protein and functions as an anti-apoptotic molecule, whereas another member of the family, Bax, serves as a proapoptotic protein. These findings have suggested that regulations of caspase-3 and Bcl-2 together with Bax inhibit the cardiac damage in myocardial infarction.

Carvacrol (CAR, 2-methyl-5-isopropylphenol), a phenolic monoterpene, is the major compound of essential oils produced by numerous aromatic plants and spices of the family Lamiaceae, which includes the genera Origanum and Thymus. It is regarded as a safe food additive and has been widely used as the chemical flavorings in candies and beverages for many years. Recently, the cumulative evidence has illustrated that CAR possesses several biological actions, which include anti-microbial, anti-inflammatory, anti-tumor and anti-oxidative activities. Two recent investigations revealed that CAR protected the liver and brain against ischemic/reperfusion (I/R) injury in vivo studies. However, there are no detailed reports regarding a potential protective effect of CAR against myocardial infarction in rats. To explore the cardioprotection of CAR may provide the therapeutic target for ischemic heart diseases. Therefore, the present study was designed to evaluate the cytoprotective property of CAR using a rat model of acute myocardial infarction as well as to further explore its potential mechanisms.

MATERIALS AND METHODS

Ethical Approval The animal experiment protocols were approved by the animal ethics committee of People’s Hospital of Zhangqiu City in China.

Animal and Acute Myocardial Infarction Production Adult male Wistar rats (250–300 g) were supplied by Beijing Animal Center (Beijing, China) and housed in polypropylene cages under a controlled environment (12:12 h light/dark cycle, 50–70% humidity, 24°C), with free access to water and food.

The model of rat acute myocardial infarction was established as previously described with minor modification. For short, rats were given an operation in sodium pentobarbitone (40 mg/kg, intraperitoneally (i.p.)) anesthesia and they were intubated and artificially ventilated with a respirator. The normal electrocardiogram (II) was obtained through a transducer attached to a multi-channel recorder (BL-420F, Cheng Du Tai Meng, China) after the electrodes were subcutaneously penetrated into four limbs. A 5-0 silk suture 1–2 mm was selected to encircle the left anterior descending coronary artery below the left atrial appendage. Sham-operated animals underwent the identical surgical procedures except for the coronary artery ligation. Efforts were made to minimize the number of animals used and their suffering. Successful ligation was confirmed by regional cyanosis of myocardial surface and ST-segment elevation.

Group Design and Drug Administration CAR (Sigma, with a purity of 98%) was dissolved in physiological saline. The chemical structure of CAR was displayed in Fig. 1.
The rats were randomly divided into five groups as follows: (1) sham-operated group (n=8), which underwent identical surgery except for the coronary artery ligation and injected with physiological saline (0.1 mL/100 g, i.p.); (2) vehicle group (n=8), which underwent the occlusion of the left coronary artery and injected with physiological saline (0.1 mL/100 g, i.p.); (3–5) CAR groups (n=8), which were subjected to the occlusion of the left coronary artery and treated with CAR 25, 50 and 100 mg/kg (i.p.), respectively. Physiological saline or CAR was injected for 7 consecutive days. Thirty minutes after the last administration, rats were operated on by occlusion of the left coronary artery.

**Measurement of Infarct Size** Six hours after the coronary artery was ligated, the hearts were immediately excised and the left ventricles were then sliced into 2 mm thick sections from the apex to the atrioventricular groove, followed by the incubation of 1% triphenyltetrazolium chloride (TTC) (Sigma-Aldrich, U.S.A.) solution at 37°C for 30 min. The brick red area indicated normal myocardium while the area without color was the ischemic heart muscles. The size of the infarcted area was calculated by the volume and weight as a percentage of the left ventricle.

**Determination of Cardiac Marker Enzymes** The blood samples were taken 6 h after the occlusion of the coronary artery, in order to determine myocardial specific enzymes, including creatine kinase (CK), MB isoenzyme of creatine kinase (CK-MB), lactate dehydrogenase (LDH) and cardiac troponin T (cTroT). The activities of CK, CK-MB and LDH were measured with the colorimetric method according to the manufacturer’s protocols (Nanjing Jiancheng Bioengineering Institute, China). Serum cTroT was measured by an immunoassay (Roche Diagnostics Elecsys 2010, Germany).

**Measurement of Malondialdehyde (MDA), Superoxide Dismutase (SOD) Glutathione (GSH), and Glutathione Peroxidase (GSH-PX) Activities** The enzymatic activities of SOD, GSH, GSH-PX and MDA in the heart homogenate were measured according to the specifications of different commercially assay kits (Nanjing Jian Cheng Bioengineering Institute, China).

The concentration of MDA, a presumptive marker of oxidant-mediated lipid peroxidation, was determined by measuring thiobarbituric-acid (TBA) reacting substances at the wavelength of 532 nm. SOD activity was estimated in the heart homogenate by calculating the rate inhibition of nucleotide oxidation. The activities of GSH-PX and GSH were assayed by quantifying the rate of oxidation of the reduced glutathione to the oxidized glutathione by H2O2. One unit of GSH-PX was represented as the amount that reduced the level of GSH by 1 μM in 1 min per milligram protein.

The content of protein in the heart homogenate was determined by means of the Coomassie brilliant blue method using bovine serum albumin as the standard.

**Assay of Caspase-3 Activity** Caspase-3 activity was measured by cleavage of chromogenic caspase substrates, acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA), a caspase-3 substrate. The amount of caspase-3 was measured with the colorimetric method using a commercial kit (Beyotime Institute of Biotechnology, China). Heart protein samples were obtained as indicated in Western blot analysis. Approximately 50 μg protein was added to a reaction buffer containing Ac-DEVD-pNA (2 mM), incubated at 37°C for 4 h, and the absorbance of yellow pNA was calculated by a spectrometer at the wavelength of 405 nm. The specific caspase-3 activity which was normalized for total protein in heart was then expressed as fold of the baseline caspase-3 activity of control group.

**Western Blot Analysis** Western blot analysis was conducted on the heart samples of each group. Briefly, heart tissues were homogenized in an ice-cold radio immunoprecipitation assay buffer containing a protease inhibitor cocktail (10 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, 1% NP-40, 5 mM ethylenediaminetetraacetic acid). After the centrifugation at 13200 × g for 20 min at 4°C, the supernatant was obtained and total protein level was measured using a standard bicinchoninic acid (BCA) method (Beyotime Institute of Biotechnology, China).

Proteins (50 μg) separated by 8% or 10% sodium dodecyl sulfate-polyacrylamide gels were transferred onto nitrocellulose membranes (Millipore, MA, USA). The membranes were blocked in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) supplemented with 5% fat-free milk for 2 h at room temperature and then incubated with a primary antibody overnight at 4°C. Primary antibodies used in the present study were: rabbit anti-caspase-3 (1:300; Santa Cruz, U.S.A.), rabbit anti-Bcl-2 (1:200, Santa Cruz, U.S.A.), rabbit anti-Bax (1:200, Santa Cruz, U.S.A.) and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:2000, Kang Chen, China). Then the membranes were rinsed and incubated with horseradish peroxidase-conjugated goat antirabbit antibody (1:5000, Santa Cruz, U.S.A.) or goat antimouse antibody (1:5000, Santa Cruz, U.S.A.) for 2 h. The detection of immunolabeled protein bands was performed using an enhanced chemiluminescence (ECL) kit (Pierce, CA, U.S.A.) and exposed on an X-ray film. GAPDH was used as a loading control. The quantification of protein band intensities was carried out by Quantity One software (BioRad, U.S.A.).

**Statistical Analysis** Data were expressed as mean ± S.D. for eight rats in each group. Experimental results were analyzed using one-way ANOVA followed by Dunnett’s test for individual comparisons between group means. All statistical analyses were generated by Statistical Package for the Social Science (SPSS) 13.0 software. A value of p<0.05 was deemed statistically significant.

**RESULTS**

**Effects of CAR on Myocardial Infarct Size in a Rat Model of Acute Myocardial Infarction** Figure 2 showed the effects of CAR on the infarct size in control and acute myocardial infarction-induced rats. It was noteworthy that the infarction size in the vehicle-treated infarcted group was 37.73 ± 1.96%. After treatment with CAR by the dose of 25, 50 and 100 mg/kg, the infarcted area was remarkably decreased.

---

Fig. 1. The Chemical Structure of CAR

\[
\text{CH}_3 \quad \text{CH}_3 \\
\text{H}_3 \text{C} \quad \text{OH} \\
\text{CH}_3 \\
\text{OH}
\]
Effects of CAR on Serum CK, CK-MB and LDH Activities together with cTnT Level in a Rat Model of Acute Myocardial Infarction The measurements of serum CK, CK-MB, LDH and cTnT in control and experimental groups were summarized in Table 1. Rats induced by acute myocardial infarction significantly exhibited higher activities of CK, CK-MB, LDH and cTnT in the serum (p<0.01) compared with the sham controls. Pretreatment with CAR at the dose of 25 (p<0.01), 50 (p<0.01) and 100 mg/kg (p<0.01) all dramatically decreased the levels of CK, CK-MB, LDH and cTnT in serum of infarcted rats versus the vehicle-treated group.

Effects of CAR on the Activities of MDA, SOD, GSH-PX and GSH in a Rat Model of Acute Myocardial Infarction Table 2 depicted the activities of MDA, SOD, GSH-PX and GSH in the heart of control and experimental rats. Rats subjected to acute myocardial infarction significantly exhibited the increase of MDA, an index of lipid peroxidation, (p<0.01) compared to that in sham controls. Administration of CAR to the infarcted rats by the dose of 25 (p<0.01), 50 (p<0.01) and 100 mg/kg (p<0.01) evidently diminished the ischemia-mediated lipid peroxidation, in comparison to the vehicle group. Furthermore, the activities of antioxidants and anti-oxidative enzymes were also measured in the heart homogenate of each group. Rats with acute myocardial infarction showed significant reductions of SOD, GSH-PX and GSH (p<0.01) versus the control rats. However, when treating with CAR by the dose of 25 (p<0.01), 50 (p<0.01) and 100 mg/kg (p<0.01), obvious elevations of SOD, GSH-PX and GSH were observed in the heart of infarcted rats than that in the vehicle group.

Effects of CAR on the Caspase-3 Activity in a Rat Model of Acute Myocardial Infarction In order to explore whether CAR could attenuate the apoptotic damage induced by myocardial infarction, the activity of caspase-3, an executioner molecule in the apoptotic signaling pathway, was determined with colorimetric analysis. As displayed in Fig. 3, caspase-3 activity in the vehicle group was markedly enhanced by 293.13% (p<0.01, n=8) compared with the sham group. In the CAR treatment (25, 50, 100 mg/kg) groups, there was an evident decline in caspase-3 activity by 36.59% (p<0.01, n=8), 43.54% (p<0.01, n=8) and 49.59% (p<0.01, n=8), respectively, compared to that in the vehicle group.

Effects of CAR on the Protein Expressions of Caspase-3, Bel-2 and Bax in a Rat Model of Acute Myocardial Infarction To corroborate the fact that CAR inhibited the cardiac damage following acute myocardial infarction in rats, Western

![Fig. 2. Effects of CAR on Infarct Size of Hearts in a Rat Model of Acute Myocardial Infarction (Mean±S.D., n=8) **p<0.01 vs. vehicle-treated group.](image1)

![Fig. 3. Effects of CAR on the Caspase-3 Activity in the Heart of Control and Acute Myocardial Infarction-Induced Rats (Mean±S.D., n=8) **p<0.01 vs. sham-operated group, ***p<0.01 vs. vehicle-treated group.](image2)

Table 1. Effects of CAR on the Activities of CK, CK-MB and LDH together with the cTnT Level in Serum of Control and Acute Myocardial Infarction-Induced Rats (Mean±S.D., n=8)

<table>
<thead>
<tr>
<th>Groups</th>
<th>CK (U/mL)</th>
<th>CK-MB (IU/L)</th>
<th>LDH (U/L)</th>
<th>cTnT (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0.25±0.05</td>
<td>79.64±3.74</td>
<td>1798.75±366.74</td>
<td>0.07±0.03</td>
</tr>
<tr>
<td>Vehicle</td>
<td>0.57±0.06**</td>
<td>188.50±6.75**</td>
<td>3649.50±418.95**</td>
<td>0.29±0.05**</td>
</tr>
<tr>
<td>CAR (25 mg/kg)</td>
<td>0.40±0.05**</td>
<td>103.23±8.17**</td>
<td>3097.75±373.14*</td>
<td>0.19±0.05*</td>
</tr>
<tr>
<td>CAR (50 mg/kg)</td>
<td>0.35±0.05**</td>
<td>89.86±7.02**</td>
<td>2583.75±360.49**</td>
<td>0.16±0.05**</td>
</tr>
<tr>
<td>CAR (100 mg/kg)</td>
<td>0.28±0.07**</td>
<td>85.19±6.38**</td>
<td>2266.13±352.26**</td>
<td>0.12±0.03**</td>
</tr>
</tbody>
</table>

**p<0.01 vs. sham-operated group, *p<0.05, ***p<0.01 vs. vehicle-treated group.

Table 2. Effects of CAR on the Activities of MDA, SOD, GSH-PX and GSH in the Heart of Control and Acute Myocardial Infarction-Induced Rats (Mean±S.D., n=8)

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA (nmol/mg protein)</th>
<th>SOD (U/mg protein)</th>
<th>GSH-PX (µg of GSH oxidized/min/mg protein)</th>
<th>GSH (mmol/g wet tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>1.67±0.22</td>
<td>24.70±1.53</td>
<td>6.31±0.62</td>
<td>35.08±2.33</td>
</tr>
<tr>
<td>Vehicle</td>
<td>5.58±0.44**</td>
<td>16.82±1.19**</td>
<td>3.27±0.21**</td>
<td>22.84±2.32**</td>
</tr>
<tr>
<td>CAR (25 mg/kg)</td>
<td>3.04±0.22**</td>
<td>20.76±1.11**</td>
<td>4.82±0.49**</td>
<td>27.72±2.59**</td>
</tr>
<tr>
<td>CAR (50 mg/kg)</td>
<td>2.76±0.26**</td>
<td>22.34±1.14**</td>
<td>5.27±0.33**</td>
<td>29.82±2.80**</td>
</tr>
<tr>
<td>CAR (100 mg/kg)</td>
<td>2.35±0.33**</td>
<td>23.08±1.15**</td>
<td>5.77±0.46**</td>
<td>32.79±2.03**</td>
</tr>
</tbody>
</table>

**p<0.01 vs. sham-operated group, *p<0.05, ***p<0.01 vs. vehicle-treated group.
 blot analysis was performed to examine the expression levels of the apoptosis-regulatory proteins including caspase-3, Bcl-2 and Bax in the heart tissue. Figure 4A demonstrated that the Western blot with caspase-3 antibody exhibited the specific bands of 20 kDa. The protein expression of caspase-3 in vehicle-treated myocardial infarction group was abundantly increased in the heart \((p<0.01)\) compared with the sham control. Nevertheless, when treatment with CAR by the dose of 25 \((p<0.01)\), 50 \((p<0.01)\) and 100 mg/kg \((p<0.01)\), a remarkable reduction of caspase-3 protein expression was noted in the infarcted rats, in comparison to the myocardial infarction-subjected group, as shown in Fig. 4B. Thus, these results by Western blot assay further confirmed that CAR treatment diminished the caspase-3 protein level in a rat model of acute myocardial infarction, which was in line with our result of caspase-3 activity measurement. Additionally, the Bcl-2 family proteins such Bcl-2 and Bax were also considered to play a pivotal role in the mediation of apoptotic cascades. Figure 4A also revealed that the checked proteins were detected in bands located at 26 kDa for Bcl-2 and 23 kDa for Bax, respectively. The quantitative analysis showed that a statistically significant reduction of Bcl-2 protein and a remarkable elevation of Bax were found in the myocardial infarction-induced rats \((p<0.01)\) compared with the control group. However, after treatment with CAR by the dose of 25 \((p<0.01)\), 50 \((p<0.01)\) and 100 mg/kg \((p<0.01)\), there were strikingly elevated expression levels of Bcl-2 and evidently decreased Bax in the heart tissue of infarcted rats, in comparison to the vehicle group, as indicated in Figs. 4C and D.

**DISCUSSION**

The results in the present study revealed for the first time that: (1) CAR reduced the infarct size and suppressed the activities of myocardial specific enzymes including CK, CK-MB, LDH and cTnT in infarcted rats; (2) a marked inhibition of the lipid peroxidation (MDA production) but remarkable increases of endogenous antioxidant enzymes (SOD and GSH) as well as the non-enzymatic scavenger (GSH-PX) were also found in a rat model of acute myocardial infarction; (3) the infarcted rats treated with CAR resulted in the dramatic decreases of caspase-3 and Bax together with the elevated Bcl-2 expression at protein level. These findings supported the notion that CAR protected hearts from acute myocardial infarction impairment and the cardioprotection of CAR might be linked with its anti-oxidative and anti-apoptotic properties. Monoterpenoid phenol derivatives derived from many plants of Lamiaceae family are widely used in clinical dentistry, agriculture and food industry. CAR is a major phenolic monoterpen and has various pharmacological actions. Our current investigation extended the therapeutic spectrum of CAR and disclosed that it was a potent cardioprotective agent on acute myocardial infarction of rats.

Infarct size and myocardial specific enzymes including CK, CK-MB, LDH and cTnT are regarded as important parameters for assessing the cardiac injury during ischemic heart diseases. Previous reports indicated the marked elevation of infarction size and CK, CK-MB and LDH activities in rats underwent acute myocardial infarction. The Serum cTnT is a very sensitive and specific indicator in detecting myocardial infarction. It is a contractile protein that is rarely found
in serum but markedly released when myocardial necrosis occurs. Consistently, our present study illustrated that the infarction size and the activities of CK, CK-MB, LDH and cTnT were evidently increased. Furthermore, they were all remarkably decreased after treating with CAR to acute myocardial infarction-induced rats, suggesting the cardioprotective effect of CAR.

It is well recognized that the excessive generation of oxygen free radicals is conceived of as a pivotal factor that exacerbates cellular damage during ischemic insult. Under physiological circumstances, the production of oxygen free radicals is usually kept under homeostatic control by endogenous antioxidant enzymes such as SOD, GSH-PX and low-molecular weight antioxidants, such as GSH. The anti-oxidative system is considered as the first line of cellular defense against oxidative injury. Several drugs, such as radical scavengers and antioxidants, have been shown to be beneficial in ischemic stroke therapy.

In the mean time, the release of lipid peroxides and aldehydes is facilitated when the cellular membrane is destroyed. MDA is formed from the break-down of fatty acids within myocardial membranes and causes a chain reaction of lipid peroxidation. Thus, MDA may be used as an index for evaluating structural oxidative injury of cell membrane. Our present investigation demonstrated that CAR treatment to the infracted rats resulted in the evident reduction of MDA and the significant elevation of SOD, GSH as well as GSH-PX. This suggested that the cardioprotection of CAR against acute myocardial infarction of rats was at least related to its anti-oxidant property.

Cardiac damage after ischemic insults occurs via oxidative stress could contribute to mitochondrial dysfunction and consequently activate an apoptotic cascade. In order to further examine the improvement of the cardiac injuries in rats caused by acute myocardial infarction after administration of CAR, we also measured the levels of apoptosis-related proteins in rat hearts. Caspases are evolutionarily conserved cysteinyl proteases with a central role in an apoptotic signaling pathway, among which caspase-3 is a key molecule in the caspase-dependent apoptotic cascade. Previous studies illustrated that caspase-3 could activate caspase-activated DNase, finally contributing to DNA fragmentation and cell loss. Cumulative investigations have proved that up-regulation of caspase-3 expression is observed after acute myocardial infarction. Results from the current study revealed that CAR treatment significantly caused the down-regulation of caspase-3 in rats subjected to acute myocardial infarction. In good line with our findings, Yu et al. also reported that CAR remarkably decreased the expression level of caspase-3 in a mouse model of focal cerebral I/R injury. In addition to caspases, Bel-2 family proteins have been also involved in the modulation of ischemia-induced apoptosis in rat myocytes. Bel-2 itself acts as the repressor of apoptosis, while another member of the family, Bax, functions as the promoter of cell death. It was previously reported myocardial infarction dramatically diminished the anti-apoptotic protein Bel-2 and increased the expression of the apoptotic promoting molecule Bax. Our findings depicted that an evident down-regulation of Bel-2 and up-regulation of Bax were noted in rats following acute myocardial infarction, which was in accordance with previous reports. However, the alterations of Bel-2 and Bax caused by ischemic damage were prevented by application of CAR. Taken together, our data showed that decreased caspase-3 and Bax as well as increased Bcl-2 proteins were found in the myocardial infarction-induced rats treated with CAR. These findings implied that CAR exerted the cardioprotective effects possibly through its anti-apoptotic profile in acute myocardial infarction of rats.

In summary, our present investigation showed that CAR attenuated the impairment caused by acute myocardial infarction of rats. The beneficial effects of CAR were likely to be modulated by its anti-oxidative and anti-apoptotic properties. This study represented the first documentation of the cardioprotective profile and its potential protective mechanism of CAR. Our results also supported the fact that CAR could be used as a promising cardioprotective agent for the treatment of acute myocardial infarction, but more detailed studies should be carried out.

REFERENCES


