Partial role of nitric oxide in infarct size limiting effect of quercetin and rutin against ischemia-reperfusion injury in normal and diabetic rats

Siva Reddy Challa*, Annapurna Akula, Sushmitha Metla & Pasumarthy N V Gopal†
Department of Pharmacology, University College of Pharmaceutical Sciences, Andhra University, Visakhapatnam 530 003, India

Received 29 June 2010; revised 20 December 2010

Reperfusion injury is remarkable clinical issue that needs to be resolved as ischemia-reperfusion is a common phenomenon encountered in numerous clinical situations. The present communication report the involvement of nitric oxide (NO) in cardioprotection offered by flavonoids (rutin and quercetin) against myocardial ischemia reperfusion. Rutin produced better cardioprotection than quercetin in normal and diabetic rats. The observed cardioprotection offered with quercetin and rutin was partially abolished by prior administration of nitric oxide synthase inhibitor, L-NAME (N-nitro-L-arginine methyl ester) in both normal and diabetic rats. L-NAME abolished the cardioprotective actions of rutin more strongly than the cardioprotective actions of quercetin. However, mechanistic study with NOS inhibitor implied the possible partial role of nitric oxide in infarct size limiting effect of quercetin and rutin

Keywords: Cardioprotection, Infarct size, Nitric oxide, Quercetin, Reperfusion injury

Reperfusion injury is an unavoidable clinical issue following thrombolytic therapy and revascularization procedures. The incidence of cardiovascular complications in normal and diabetic patients has spurred the research efforts to design the therapeutic modalities that limits reperfusion injury and maximizes the beneficial outcomes of reperfusion therapy. Substantial epidemiological\(^1\) and experimental\(^2\) evidences support the cardioprotective effects of flavonoids. It was believed that cardioprotective actions of flavonoids against ischemia reperfusion injury are mostly attributed to their antioxidant effects. However, antioxidant effects of flavonoids, quercetin and rutin are not enough to explain the extent of cardioprotection offered by these drugs\(^3\). It is important to explore other mechanisms ascribed to the cardioprotection of quercetin and rutin against ischemia-reperfusion (IR) injury. Flavonoids are known to stimulate endothelial nitric oxide synthase (e NOS) and hence augment the formation and release of nitric oxide (NO)\(^4\) thereby reverse endothelial dysfunction\(^5\). Nitric oxide (NO) attenuates the reperfusion injury by improving endothelial function, depressing platelet and neutrophil activation, and augmenting coronary flow.

In view of declined basal NO release following ischemia reperfusion\(^6\) and the ability of flavonoids to modulate the NO formation and release in various other experimental models, the present study aims to investigate the role of nitric oxide in the cardioprotective mechanisms offered by flavonoids, quercetin and rutin in ischemia reperfusion induced myocardial infarction in normal and streptozotocin (STZ) induced diabetic rats.

Materials and Methods

Drugs and chemicals—Streptozotocin (STZ), quercetin, rutin and N\(\omega\)-nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma Chemical Company, St. Louis, USA. 2, 3, 5-triphenyl tetrazolium chloride (TTC) was purchased from BDH Chemicals Ltd, England. Thiopentone sodium was supplied by Abbott Lab Ltd, Ankleswar, India.

Animals—Albino Wistar rats (National Institute of Nutrition, Hyderabad, India) of either sex weighing 200-250 g were used. Animals were maintained under standard laboratory conditions at 25\(^\circ\)± 2\(^\circ\)C, 50 ± 15 % RH and normal photoperiod (12 h dark/ 12 h light).
Commercial pellet diet (Rayon’s Biotechnology Pvt Ltd, India) and water were provided ad libitum. The experimental protocol was approved by the Institutional Animal Ethics Committee and by the Animal Regulatory Body of the Government (Regd. No. 516/01/A/CPCSEA).

Induction of diabetes—Diabetes was induced by a single (i.v.) injection of streptozotocin (STZ) 45 mg/kg of body weight, dissolved in citrate buffer (pH 4.5) into the tail vein of animals lightly anaesthetized with ether. Age-matched rats were injected with citrate buffer only. Diabetes was confirmed by estimations made after third day of STZ injection for serum glucose by an Auto analyzer (Dade Behring, USA). Following two weeks of diabetes induction, rats were subjected to surgical procedure. About 20% of mortality was observed in diabetic rats even before being subjected to ischemia-reperfusion injury. Diabetic condition was confirmed by the measurement of serum glucose levels. Diabetic rats showing serum glucose levels more than 350 mg/dl were used for the experiment.

Surgical procedure—Rats were anaesthetized with thiopentone sodium (30 mg/kg, ip), tracheotomized and ventilated with room air by a Techno positive pressure mechanical respirator (Animal Respirator, Crompton Parkinson Ltd., UK). The right jugular vein was cannulated in order to inject drugs. A left thoracotomy (an incision into the pleural space of the chest) and pericardiotomy (incision into the pericardium) were performed, and the left coronary artery was dissected free above the first diagonal branch and was ligated just below the origin of left circumflex artery with the help of a silk thread. The artery was occluded for 30 min by a knot. The silk thread was removed after 30 min with the help of two-knot releasers to allow reperfusion of the heart for succeeding 4 h.

Quantification of infarct size—In all the groups after sacrificing the animal by giving excess anaesthesia, the heart was excised from the thorax rapidly and the greater vessels were removed. The left ventricle was separated from the heart and weighed. It was sliced parallel to the atroventricular groove into 2-3 mm thick sections and the slices were incubated in 1% TTC solution prepared in phosphate buffer, pH 7.4 for 30 min at 37°C. In viable myocardium, TTC is converted by dehydrogenases to a red formazan pigment that stains tissue dark red. The infarcted myocardium that does not take TTC stain where the dehydrogenases were drained off, remained pale in color, the pale necrotic tissue was separated from the stained portions and weighed on an electronic balance (Dhona 200D, Dhona Instruments Ltd, India). Infarct size was calculated as a percentage fraction of non-viable myocardium of the left ventricle.

Experimental design
Protocol I: The normal rats were randomly divided into following 13 groups of 6 animals each: Gr.1, normal sham control group; Gr.2, normal control ischemia-reperfusion (I/R) group treated with saline; Gr.3, treated with L-NAME (10 mg/kg); Gr.4, treated with vehicle (DMSO) control group; Gr.5, treated with DMSO (0.2 ml) and L-NAME (10 mg/kg); Gr.6, treated with quercetin (5 mg/kg); Gr.7, treated with quercetin (5 mg/kg) and L-NAME (10 mg/kg); Gr.8, treated with quercetin (10 mg/kg); Gr.9, treated with quercetin (10 mg/kg) and L-NAME (10 mg/kg); Gr.10, treated with rutin (5 mg/kg); Gr.11, treated with rutin (5 mg/kg) and L-NAME (10 mg/kg); Gr.12, treated with rutin (10 mg/kg); Gr.13, treated with rutin (10 mg/kg) and L-NAME (10 mg/kg).

Protocol II: The STZ induced diabetic rats were randomly divided into 13 diabetic groups of 6 animals each. The details of group were same as in protocol I (normal rats) but in protocol II, diabetic rats were used.

L-NAME was dissolved in saline and administered as iv bolus injection through the jugular vein 10 min before coronary artery occlusion to allow adequate time for inhibition. Quercetin and rutin were dissolved in DMSO and administered ip 10 min before reperfusion.

Statistical analysis—The results are expressed as mean±SD. Differences in infarct size were determined by factorial one-way analysis of variance. Individual groups were compared using Tukey’s test. Differences with P<0.05 were considered statistically significant.

Results
Effect of quercetin and rutin on infarct size with and without NO synthase inhibitor (L-NAME) in both normal and diabetic rats—Results of both normal rats and diabetic rats are presented in Table 1. Cardiac damage was observed in the control group animals compared to sham operated group and the damage increased in diabetic control animals compared to normal control rats subjected to IR injury.
Infarct size obtained with the administration of L-NAME (NO synthase inhibitor) was almost similar to that of infarct size observed in normal control group animals. There was no significant difference between infarct sizes of L-NAME treated group and normal control group. The similar result was observed in the diabetic rats also. Therefore, in the present study, L-NAME by itself did not increase the infarct size in both normal and diabetic rats.

In vehicle (DMSO, dimethyl sulfoxide) treated normal and diabetic animals, infarct size was slightly but significantly reduced. This is probably due to known antioxidant activity of DMSO. Significant cardioprotection with quercetin treatment (5, 10 mg/kg) and rutin treatment (5, 10 mg/kg) was observed in terms of limiting the infarct size in normal and diabetic rats. It is quite interesting to note that rutin offered better cardioprotective action than quercetin in both normal and diabetic rats. Particularly, quercetin showed moderate cardioprotective action in diabetic rats where as rutin produced better efficacy than quercetin.

The cardioprotection obtained by the quercetin (5 and 10 mg/kg) was significantly but partially blocked by the L-NAME in both normal and diabetic rats. Similarly, the cardioprotection obtained by the rutin (5 and 10 mg/kg) was significantly but partially blocked by the NO synthase inhibitor (L-NAME) in both normal and diabetic rats.

L-NAME antagonized the cardioprotective actions of rutin more effectively than quercetin actions in both normal and diabetic rats. Blockade of cardioprotective actions of quercetin and rutin by L-NAME was more in diabetic rats when compared to normal rats.

### Table 1—Effect of quercetin and rutin on percentage left ventricle necrosis (PLVN) with and without L-NAME in ischemia reperfusion injury in both normal and diabetic rats

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Treatment</th>
<th>PLVN (%)</th>
<th>Normal rats</th>
<th>Diabetic rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>Sham operated group</td>
<td>4.89 ± 0.39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.04 ± 1.56&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Group 2</td>
<td>Control I/R</td>
<td>50.49 ± 1.40</td>
<td>58.73 ± 1.29</td>
<td></td>
</tr>
<tr>
<td>Group 3</td>
<td>Control I/R with L-NAME</td>
<td>50.40 ± 1.14</td>
<td>57.41 ± 0.82</td>
<td></td>
</tr>
<tr>
<td>Group 4</td>
<td>Vehicle control I/R</td>
<td>47.96 ± 0.74&lt;sup&gt;a&lt;/sup&gt;</td>
<td>56.22 ± 1.64&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Group 5</td>
<td>Vehicle control I/R with L-NAME</td>
<td>50.30 ± 0.80</td>
<td>56.59 ± 1.13</td>
<td></td>
</tr>
<tr>
<td>Group 6</td>
<td>Quercetin (5 mg/kg)</td>
<td>21.91 ± 0.80&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>44.28 ± 0.88&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Group 7</td>
<td>Quercetin (5 mg/kg)+ L-NAME (10 mg/kg)</td>
<td>41.84 ± 0.98&lt;sup&gt;b&lt;/sup&gt;</td>
<td>54.46 ± 0.65&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Group 8</td>
<td>Quercetin (10 mg/kg)</td>
<td>9.69 ± 0.83&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>28.38 ± 1.55&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Group 9</td>
<td>Quercetin (10 mg/kg)+ L-NAME (10 mg/kg)</td>
<td>23.51 ± 0.48&lt;sup&gt;d&lt;/sup&gt;</td>
<td>43.93 ± 0.5&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Group 10</td>
<td>Rutin (5 mg/kg)</td>
<td>22.66 ± 0.58&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>28.04 ± 1.53&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Group 11</td>
<td>Rutin (5 mg/kg)+ L-NAME (10 mg/kg)</td>
<td>47.53 ± 0.66&lt;sup&gt;d&lt;/sup&gt;</td>
<td>49.85 ± 0.68&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Group 12</td>
<td>Rutin (10 mg/kg)</td>
<td>2.91 ± 0.58&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>7.45 ± 0.85&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Group 13</td>
<td>Rutin (10 mg/kg)+L-NAME (10 mg/kg)</td>
<td>26.91 ± 0.59&lt;sup&gt;d&lt;/sup&gt;</td>
<td>36.00 ± 0.50&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

*P<0.05, vs a normal control I/R, b vehicle control I/R, c normal control treated with quercetin (5 mg/kg), d normal control treated with quercetin (10 mg/kg), e normal control treated with rutin (5 mg/kg), f normal control treated with rutin (10 mg/kg), g diabetic control I/R, h diabetic vehicle control I/R, i diabetic control treated with quercetin (5 mg/kg), j diabetic control treated with quercetin (10 mg/kg), k diabetic control treated with rutin (5 mg/kg), and l diabetic control treated with rutin (10 mg/kg)

Discussion

The present study demonstrated significant increase in infarct size in normal control rats subjected to ischemia-reperfusion compared to sham operated group. Myocardial damage observed in control animals probably involves multiple mechanisms like free radical induced oxidative damage, neutrophil accumulation with “no reflow phenomenon” and declined bioavailability of NO were clearly evident during diabetes.

There was no significant difference in the infarct size between L-NAME treated group and control group. In the present study, L-NAME by itself did not increase infarct size. This may be related to the fact that eNOS inhibitor blocks NO synthesis. It also blocks super oxide formation. Normally there is a balance between the protective effect of NO and the harmful effect of the free radical.

In vehicle (DMSO, dimethyl sulfoxide) treated normal and diabetic animals, infarct size was slightly
but significantly reduced. This is probably due to known anti-oxidant activity of DMSO. Flavonoids, quercetin and rutin offered significant and dose dependent cardioprotection in terms of limiting the infarct size in both normal and diabetic rats. Rutin exerted the better activity than quercetin in limiting the infarct size. It could probably be due to better absorption of rutin in comparison to quercetin. Naturally occurring flavonols exist predominantly in a glycolysated form e.g. rutin rather than in their aglycon form. The forms of the flavonoids seem to influence the rate of absorption. Hollman et al13 suggested that the glycolysated forms of quercetin are absorbed more readily than the aglycone form, except in case of catechin, which is not glycosylated in nature, is absorbed relatively efficiently14.

Administration of L-NAME prior to the administration of quercetin and rutin, partially abolished the cardioprotection afforded by quercetin and rutin in both normal and diabetic rats. This suggests the partial role of NO in the cardioprotective mechanisms of quercetin and rutin. These flavonoids probably act in part by increasing activity of nitric oxide synthase resulting in enhanced NO synthesis. Indeed, polyphenols have been shown to increase the formation of NO by endothelial nitric oxide synthase15. An earlier study also demonstrated an increased activity of eNOS in rat aortas after a flavonoid rich diet 16.

In conclusion, the possible partial involvement of NO in the beneficial effects of flavonoids, quercetin and rutin against ischemia-reperfusion injury in normal and diabetic rats was observed. Since it is a mechanistic study with NO synthase inhibitor, this mechanism has to be confirmed with further studies where one can estimate NO levels and eNOS expression and its levels in myocardium.

Acknowledgement
Authors are thankful to the Department of Pharmacology, University College of Pharmaceutical Sciences, Andhra University and Visakhapatnam for providing research facilities.

References