Enhanced cardioprotection against ischemia-reperfusion injury with a dipyridamole and low-dose atorvastatin combination

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Ye Y, Lin Y, Perez-Polo R, Huang MH, Hughes MG, McAdoo DJ, Manickavasagam S, Uretsky BF, Birnbaum Y. Enhanced cardioprotection against ischemia-reperfusion injury with a dipyridamole and low-dose atorvastatin combination. Am J Physiol Heart Circ Physiol 293: H813–H818, 2007. First published April 6, 2007; doi:10.1152/ajpheart.00210.2007.— Atorvastatin (ATV) limits infarct size (IS) by activating Akt and eNOS (48.1–50%), whereas ATV and DIP (6 mg·kg−1·day−1) did not affect myocardial Ser473 phosphorylated-Akt (3.1 pg/ml) and DIP (51.5 pg/ml) and DIP (45.8 pg/ml). Rats underwent 30 min of myocardial ischemia followed by 4 h of reperfusion (IS protocol), or hearts were explanted for immunoblotting. As a result, IS in the controls was 34.0 ± 2.8% of the area at risk. ATV (33.1 ± 2.1%) and DIP (30.5 ± 1.5%) did not affect IS, whereas ATV + DIP reduced IS (12.2 ± 0.5%; P < 0.001 vs. each of the other groups). There was no difference in IS between the Ami alone (48.1 ± 0.8%) and the Ami + ATV + DIP (45.8 ± 2.9%) group (P = 0.422), suggesting that Ami completely blocked the protective effect. Myocardial adenosine level in the controls was 30.6 ± 3.6 µg/µL. ATV (51.0 ± 4.9 µg/µL) and DIP (51.5 ± 6.8 µg/µL) caused a small increase in adenosine levels, whereas ATV + DIP caused a greater increase in adenosine levels (66.4 ± 3.1 µg/µL). ATV and DIP alone did not affect myocardial Ser473 phosphorylated-Akt and Ser1177 phosphorylated-eNOS levels, whereas ATV + DIP significantly increased them. In conclusion, low-dose ATV and DIP had synergistic effects in reducing myocardial IS and activation of Akt and eNOS. This combination may have a potential benefit in increasing the eNOS-mediated pleiotropic effects of statins.

Adenosine; Akt; infarct size; nitric oxide synthase

SEVERAL EXPERIMENTAL STUDIES have shown that hydroxymethyl glutaryl coenzyme A reductase inhibitors (statins), administered either before myocardial ischemia (3, 6, 10, 23, 24, 34–36, 43, 44, 48, 50–53) or immediately after reperfusion (5, 12, 50), reduce myocardial infarct size (IS). It has been shown that statins activate phosphatidylinositol-3-kinase (5, 12, 19, 50) with subsequent activation of both Akt (5, 11, 12, 18, 19, 27, 30, 35, 38, 50, 53) and ecto-5-nucleotidase, which generates adenosine (9, 35, 46). Both Akt (5, 18, 27) and adenosine (15, 29, 39, 40, 47) activate endothelial nitric oxide synthase (eNOS). eNOS activation is essential for this protective effect, since nonspecific nitric oxide synthase inhibitors blunt the IS-limiting effect of statins (6, 51) and since statins do not reduce IS in eNOS−/− mice (5, 23, 32). Inhibition of the adenosine receptors, on the other hand, also abrogates the IS-limiting effects of statins (28, 35). We recently showed that adenosine is needed for phosphorylation of Akt by 3-phosphoinositide-dependent kinase-1 and subsequent phosphorylation of eNOS (54).

Dipyridamole (DIP) blocks the cellular reuptake of adenosine, thus increasing interstitial adenosine concentrations (17, 37). When administered intravenously at high doses (i.e., 0.56 mg/kg over 4 min), DIP may exacerbate myocardial ischemia by diverting blood flow from the diseased vessels with fixed narrowings to healthier branches undergoing vasodilatation (1). However, at low doses (300–400 mg/day) oral DIP has a small hemodynamic and significant antiplatelet effects and has been used in the clinical setting to prevent cardiovascular and cerebrovascular events (37).

DIP alone has no effect on myocardial IS, but it does augment the effects of ischemic preconditioning (22, 31, 42, 45) since adenosine is one of the triggers of preconditioning (16).

We asked whether DIP and atorvastatin (ATV) have synergistic effects on augmenting myocardial protection by increasing the myocardial adenosine levels.

METHODS

Animal care. All animals received humane care in compliance with The Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (Publication No. 85-23, Revised 1996). The protocol was approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee. Experiments were conducted on male Sprague-Dawley rats.

Materials. We used crushed tablets of ATV (Lipitor, Pfizer). DIP (an adenosine reuptake inhibitor) was purchased from Sigma (St. Louis, MO) and aminophylline (Ami, a nonselective adenosine receptor inhibitor) from American Regent (Shirley, NY). Monoclonal anti-eNOS antibodies were purchased from BD Bioscience (San Jose, CA) and monoclonal anti-β-actin antibody from Sigma (St. Louis, MO). Anti-Akt antibodies, anti-Ser473 phosphorylated (p)-Akt antibodies, and anti-Ser1177 p-eNOS antibodies were purchased from Cell Signaling (Beverly, MA).

Treatment. In protocol 1, rats received a 3-day pretreatment with water alone, ATV (2 mg·kg−1·day−1), DIP (6 mg·kg−1·day−1), or ATV + DIP. ATV was administered by oral gavage once daily. DIP was added to the drinking water at a concentration of 0.6 mg/ml. On September 9, 2017 http://ajpheart.physiology.org/ Downloaded from http://ajpheart.physiology.org/
the fourth day, rats underwent coronary artery ligation for 30 min followed by 4 h of reperfusion (IS protocol), or hearts were harvested under deep anesthesia for immunoblotting and a determination of tissue adenosine levels.

In protocol 2, rats received a 3-day pretreatment with Ami (10 mg·kg\(^{-1}\)·day\(^{-1}\)) or Ami + ATV (2 mg·kg\(^{-1}\)·day\(^{-1}\)) + DIP (6 mg·kg\(^{-1}\)·day\(^{-1}\)). Ami was added to the drinking water at a concentration of 1 mg/ml. On the fourth day, rats underwent coronary artery ligation for 30 min followed by 4 h of reperfusion (IS protocol), or hearts were harvested under deep anesthesia for immunoblotting.  

**IS surgical protocol.** The rat model of myocardial ischemia-reperfusion injury has been described in detail (3, 6, 10, 53). On the fourth day, rats were anesthetized with an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (6 mg/kg), intubated, and ventilated (fractional inspired O\(_2\) = 30%). The rectal temperature was monitored, and body temperature was maintained between 36.7° and 37.3°C throughout the experiment. The left carotid artery was cannulated. The chest was opened, and the left coronary artery was incised with a suture and ligated for 30 min. Isoflurane (1–2.5% titrated to effect) was added after the beginning of ischemia to maintain performance liquid chromatography (HPLC) according to the procedure of Wojcik and Neff (49). Myocardial samples were homogenized with ~10 volumes of 0.25 M ZnSO\(_4\). The protein concentration was determined by Lowry assay. Ten volumes of 0.25 M BaOH\(_2\) were added, and samples were centrifuged at 30,000 g for 10 min. Supernatants were then transferred to a conical tube, and 5 μl chloroacet-aldehyde were added to 20 μl of supernatant. Tubes were capped, mixed, and submerged in boiling water for 10 min. The samples were analyzed by HPLC using a Waters C18 reversed-phase 150 mm × 4.6 mm column. The mobile phase was a 50 mM acetate buffer (pH 4.5) and 6.5% aqueous acetonitrile (vol/vol) with 2 mM sodium octyl sulfonate dissolved in it. The flow rate was 1.1 ml/min. The excitation monochromator was set to a wavelength of 270 nm, and the cutoff wavelength of the emission filter was 389 nm.

**Immunoblotting.** Myocardial samples from the LV wall were homogenized in RIPA lysis buffer (Santa Cruz Biotechnology) and centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant was collected, and the total protein concentration was determined using the Lowry protein assay. Protein samples with loading buffer were run in 4–20% Tris·HCl Ready Gel at a 100 V for 2 h until the desired molecular weight bands were separated. After electrophoresis, the gel was equilibrated in transfer buffer (25 mM Tris, 193 mM glycine, 0.1% SDS, and 10% methanol), and the proteins were transferred to a nitrocellulose membrane. Protein signals were quantified by an image-scanning densitometer, and the strength of each protein signal was normalized to the corresponding β-actin stain signal. Data are expressed as a ratio between the protein and the corresponding β-actin signal density.

### Table 1. Protocol 1: body weight, left ventricular weight, area at risk, and infarct size of rats

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Control</th>
<th>ATV</th>
<th>DIP</th>
<th>ATV + DIP</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td>251±2</td>
<td>249±3</td>
<td>248±2</td>
<td>251±3</td>
<td>0.055</td>
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<td>Left ventricular weight, mg</td>
<td>1,098±34</td>
<td>1,129±8</td>
<td>1,085±32</td>
<td>1,136±6</td>
<td>0.485</td>
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<tr>
<td>Area at risk, % of LV</td>
<td>37.5±2.5</td>
<td>37.9±2.5</td>
<td>38.7±1.1</td>
<td>34.4±0.7</td>
<td>0.463</td>
</tr>
<tr>
<td>Infarct size, % of LV</td>
<td>12.4±0.8</td>
<td>12.6±1.1</td>
<td>11.8±0.6</td>
<td>4.2±0.2*</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of rats. ATV, atorvastatin; DIP, dipyridamole; LV, left ventricle. *P < 0.001 vs. control.
Statistical analyses. Data are expressed as means ± SE. Comparisons among the groups were performed by one-way ANOVA with Dunnett’s correction for multiple comparisons (SPSS, version 14.0). The differences in heart rate and mean blood pressure were compared using two-way repeated-measures ANOVA with Holm-Sidak multiple comparison procedures. Values of \( P < 0.05 \) were considered statistically significant.

The authors had full access to the data and take responsibility for its integrity. All authors have read and approved this study.

RESULTS

Infarct size. In protocol 1, a total of 33 rats were included. Two rats died during coronary artery occlusion (one in the control group and one in the ATV/DIP group). Body weight, LV weight, and AR were comparable among groups (Table 1). IS, expressed either as a percentage of the LV (Table 1) or as a percentage of the AR (IS) (Fig. 1), was significantly smaller in the ATV/DIP group (12.2 ± 0.5% of the AR) compared with the other three groups. IS in the control group was 34.0 ± 2.8% of the AR. ATV at 2 mg·kg\(^{-1}\)·day\(^{-1}\) alone (33.1 ± 2.1% of the AR) and DIP alone at 6 mg·kg\(^{-1}\)·day\(^{-1}\) (30.5 ± 1.5% of the AR) did not affect IS. There was no treatment effect on heart rate (\( P = 0.224 \)) or mean blood pressure (\( P = 0.062 \)), although there was a significant time effect, i.e., significant differences between preocclusion, occlusion, and reperfusion (\( P < 0.001 \) for both heart rate and blood pressure).

![Graph showing myocardial adenosine levels](image)

Fig. 3. Myocardial adenosine levels. Overall, there were significant differences among groups (\( P = 0.002 \)). ATV alone (\( P = 0.028 \)) and DIP alone (\( P = 0.025 \)) caused a small increase in myocardial adenosine levels compared with levels in the control (Cont) group. Myocardial adenosine levels were significantly higher in the ATV + DIP group than in the control group (\( P = 0.001 \)).

![Graph showing Akt and eNOS expression](image)

Fig. 4. Representative immunoblots and densitometric analyses of myocardial levels of total Akt (A and B), total endothelial nitric oxide synthase (eNOS; C and D), phosphorylated (p)-Akt (E and F), and p-eNOS (G and H) are shown (protocol 1). ATV and DIP, alone or in combination, did not affect total Akt and eNOS expression. Although ATV alone and DIP alone did not affect p-Akt and p-eNOS levels, their combination resulted in a significant increase in p-Akt and p-eNOS levels. *\( P < 0.05 \) vs. control; †\( P < 0.05 \) vs. DIP; ‡\( P < 0.05 \) vs. ATV.
In protocol 2, a total of 12 rats was included. One rat treated with ATV + DIP + Ami died during the reperfusion period. Body weight (251 ± 1 vs. 251 ± 1 g; P = 0.817), LV weight (1,172 ± 16 vs. 1,246 ± 80 mg; P = 0.345), and AR (35.1 ± 0.4% vs. 35.8 ± 1.6% of the LV; P = 0.662) were comparable between the Ami and the ATV + DIP + Ami groups. IS was comparable between the groups (16.9 ± 0.4% vs. 16.4 ± 1.1% of the LV; P = 0.623). Figure 2 shows IS (as a percentage of AR) in the two groups. There were no significant differences between the two groups in heart rate (P = 0.108) or mean blood pressure (P = 0.5), although there was a significant time effect, i.e., significant differences between preconditioning, occlusion, and reperfusion (P < 0.001 for both heart rate and blood pressure).

Myocardial adenosine levels. Myocardial adenosine levels in the control group were 30.6 ± 3.6 pg/μl. ATV alone caused a small increase in myocardial adenosine levels (51.0 ± 4.9 pg/μl; P = 0.028) (Fig. 3). DIP alone also caused a small increase in myocardial adenosine levels (51.5 ± 6.8 pg/μl; P = 0.025). In contrast, myocardial adenosine levels were much higher in the ATV + DIP group than in the control group (66.4 ± 3.1 pg/μl; P = 0.001).

Immunoblotting. ATV and DIP, alone or in combination, did not affect myocardial total Akt (Fig. 4, A and B) or total eNOS (Fig. 4, C and D) levels. ATV alone (99.39 ± 4.71%) and DIP alone (103.34 ± 3.76%) did not significantly affect p-Akt (Fig. 4, E and F) and p-eNOS (99.30 ± 6.18% and 110.48 ± 2.42%, respectively) (Fig. 4, G and H) levels. However, the combination of ATV + DIP caused a significant increase in myocardial p-Akt (255.84 ± 11.11%) and p-eNOS levels (473.66 ± 23.65%).

Ami completely blocked the ATV + DIP induction of Akt and eNOS phosphorylation (Fig. 5).

DISCUSSION

We have shown that DIP and ATV have synergistic effects on myocardial protection against ischemia-reperfusion injury mediated by augmentation of myocardial adenosine levels, leading to enhanced Akt and eNOS phosphorylation. The protective effect of ATV + DIP was completely abrogated with Ami. Moreover, Ami completely blocked the induction of Akt and eNOS phosphorylation by ATV + DIP. These data support the essential role of adenosine in mediating the protective effect of ATV + DIP.

Although several investigators have shown that statin pretreatment limits IS (3, 6, 10, 23, 24, 34–36, 43, 44, 48, 50–53), high oral doses are needed to achieve maximal cardioprotection. For example, a 3-day pretreatment with oral ATV at 10 and 75 mg·kg⁻¹·day⁻¹ reduces IS, whereas at 2 mg·kg⁻¹·day⁻¹ ATV alone does not affect IS (6). Also, a 3-day pretreatment with ATV at 1 mg·kg⁻¹·day⁻¹ does not affect myocardial levels of p-eNOS or calcium-dependent nitric oxide synthase activity and does not limit IS (34). Thus, to achieve maximal protection, high doses of statins are needed. Another confound is that although blood levels of ATV 16 h after a third dose of ATV (10 mg·kg⁻¹·day⁻¹) in the rat are comparable with those in humans treated with ATV at 80 mg/day (8), not all patients can tolerate maximal doses of statins. Thus it is useful to assess the efficacy of drug combinations that enhance the protective effect and decrease of IS. It is known that sildenafil augments the IS-limiting effect of statins (34), whereas selective cyclooxygenase-2 inhibitors (3, 10) and aspirin (7) attenuate it. Since Akt (5, 11, 12, 18, 19, 27, 30, 35, 38, 50, 53) and eNOS (2, 5, 13, 23, 52) activation plays an important role in statin protection from ischemia-reperfusion injury and since statins activate ecto-5-nucleotidase and increase tissue levels of adenosine (9, 35, 46), which in turn activates Akt (26) and eNOS (15, 29, 39, 40, 47), we asked whether ATV, which generates adenosine via ecto-5-nucleotidase activation (9, 35, 46), and DIP, which prevents the reuptake and degradation of adenosine (17, 37), could have synergistic effects on eNOS activation and IS limitation.

It is known that although acute administration of DIP does not affect IS, it potentiates the protective effect of ischemic preconditioning (22, 31, 42, 45) and adenosine (4). Others have shown this in guinea pigs (14) and humans (20, 21, 32, 33, 41).
DIP alone may be protective. For example, Figueredo et al. (14) showed that oral DIP at 4 mg·kg$^{-1}$·day$^{-1}$ for 6 wk was cardioprotective in guinea pigs (14). Riksen reported that at 200 mg/day for 1 wk DIP ameliorated ischemia-reperfusion injury in forearm skeletal muscle of normal volunteers (33). Kitakaze et al. (25) showed that oral DIP (300 mg/day) for 6 mo ameliorated the severity of heart failure in patients. Several studies have shown that intracoronary administration of DIP at 0.5 mg/kg before percutaneous transluminal angioplasty reduced the severity of chest pain and the magnitude of ST deviation and ameliorated the decrease in LV systolic and diastolic dysfunction during balloon inflation (20, 21, 41).

Many patients who are at risk or have established cardiovascular disease are on antiplatelet therapy, mainly aspirin (alone or in combination with clopidogrel or DIP). The majority of these patients are also receiving concomitant statin treatment. The combination of DIP, an agent with antiplatelet properties (37), with statins is especially appealing given that aspirin attenuates the IS-limiting effects of ATV in a dose-dependent way in the rat (7). Thus, adding DIP may augment the pleiotropic effects of statins that are mediated by eNOS activation, including vasodilatation and protection against ischemia-reperfusion injury, and thus enable us to lower the aspirin dose. Further studies are needed to confirm that the synergistic effects of DIP and statins are persisting over time and that this combination enhances the anti-inflammatory and antiatherosclerotic effects of statins. Moreover, the optimal DIP dose should be determined. The current recommended oral dose of DIP for risk reduction of thrombotic events is 300–400 mg/day (37). It might be that with higher doses and/or longer duration of therapy, DIP alone could have a protective effect and that the effects of the combination treatment could be greater.

REFERENCES


Dipyridamole and Low-Dose Atorvastatin


