Aspirin before reperfusion blunts the infarct size limiting effect of atorvastatin

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Submitted 20 November 2006; accepted in final form 1 February 2007

Birnbaum Y, Lin Y, Ye Y, Martinez JD, Huang M-H, Lui CY, Perez-Polo JR, Uretsky BF. Aspirin before reperfusion blunts the infarct size limiting effect of atorvastatin. Am J Physiol Heart Circ Physiol 292: H2891–H2897, 2007. — We assessed whether aspirin (acetylsalicylic acid, ASA), administered before reperfusion, abrogates the infarct size (IS)-limiting effect of atorvastatin (ATV). Statins reduce IS. This dose-dependent effect is mediated by upregulation of cyclooxygenase-2 (COX2) and PGI2 production. Administration of selective COX2-inhibitors either with ATV for 3 days or immediately before coronary occlusion blocks the IS-limiting effect of ATV. Sprague-Dawley rats received 3-day ATV (10 mg·kg−1·day−1) or water alone. Rats underwent 30 min coronary artery occlusion and 4 h reperfusion (IS protocol, n = 8 in each group), or rats underwent 30 min coronary artery occlusion and 10 min reperfusion (enzyme expression and activity protocol, n = 4 in each group). Immediately before reperfusion rats received intravenous ASA (5, 10, or 20 mg/kg) or saline. Area-at-risk (AR) was assessed by triphenyltetrazolium chloride. ATV reduced IS (29.0 ± 10.1% of AR) compared with controls (31.0 ± 1.4%). ASA dose dependently blocked the upregulation of COX2 by ATV. COX2 activity was as follows: control, 8.93 ± 0.90 pg/mg; ATV, 75.85 ± 1.08 pg/mg; ATV + ASAs, 34.39 ± 1.48 pg/mg; ATV + ASA10, 19.87 ± 1.10 pg/mg; and ATV + ASA20, 9.36 ± 0.94 pg/mg. ASA, administered before reperfusion in doses comparable to those used in the clinical setting, abrogates the IS-limiting effect of ATV in a model with mechanical occlusion of the coronary artery. This potential adverse interaction should be further investigated in the clinical setting of acute coronary syndromes.

acetylsalicylic acid; cyclooxygenase-2

ASPIRIN (acetylsalicylic acid, ASA) is an essential part of treatment of patients with ST elevation acute myocardial infarction. The Second International Study of Infarct Survival (ISIS-2) has shown that ASA at 160 mg/day for 1 mo was associated with a reduction in 5-wk cardiovascular mortality to a similar extent as streptokinase infusion (28a). The American College of Cardiology/American Heart Association guidelines for the management of patients with ST elevation acute myocardial infarction recommend prompt administration of chewed ASA at 160–325 mg within 10 min of presentation of chest pain (1). The European Society of Cardiology guidelines also recommend an alternative intravenous route (250 mg) for patients who cannot swallow ASA (33). However, many centers are using higher doses (intravenous 500 mg) of ASA (12). For example, in the Intracoronary Stenting and Antithrombotic Regimen: Rapid Early Action for Coronary Treatment 2 (ISAR-REACT-2) trial, 500 mg of ASA were administered either orally or intravenously (19). It is commonly believed that at low doses, ASA is more specific for cyclooxygenase (COX)-1 (COX1) than cyclooxygenase-2 (COX2), based on an in vitro study showing that higher concentration of ASA were needed for inhibition of PGE2 production than for thromboxane B2 production in whole blood samples from healthy subjects (9). However, other studies did not report such selectivity (24, 34). Another explanation suggested by de Gaetano et al. (11) is that oral ASA acetylates platelet COX1 mainly in the portal circulation. Because ASA undergoes extensive first-pass deacetylation within the enterohepatic circulation, systemic acetylation of COX is minimal, at least with low doses of oral ASA (11). In contrast, when chewed or administered intravenously, higher systemic blood levels are achieved and, consequently, inhibition of the vascular COX is expected.

Shimura et al. (30) have shown that oral ASA at 25 mg/kg, but not at 5 or 10 mg/kg, abrogated the protective effect of the late phase of ischemic preconditioning in the rabbit. Moreover, Gross et al. (14) have found that when given intravenously, much lower doses of ASA (1 and 3 mg/kg) administered before reperfusion abolished the infarct size (IS)-limiting effect of morphine in the rat. There is growing evidence that interventions during reperfusion determine final IS by affecting “reperfusion injury” (10).

The use of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) for primary and secondary prevention of cardiovascular disease has increased over the last few years (13a). There are data supporting beneficial effects of statins in the early stages of acute myocardial infarction in humans (8, 28, 32). Recently, we have shown that 3-day pretreatment with atorvastatin (ATV) (10 mg·kg−1·day−1) limit IS in the rat (3, 4, 7, 36). The IS-limiting effect of ATV was abrogated when specific COX2 inhibitors were co-administered with atorvastatin for 3 days (7) or when given intravenously just before coronary artery occlusion (3), suggesting that the protective effect of ATV is mediated via COX2. Thus it might be that chewable or intravenous ASA could abrogate the direct myocardial protective effect of statins. This potential adverse effect may offset the favorable anti-platelet effects of ASA. Therefore, we have investigated whether intravenous ASA affects the IS-limiting effect of ATV pretreatment.

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METHODS

Animal Care

Male Sprague-Dawley rats received humane care in compliance with The Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996). The protocol was approved by University of Texas Medical Branch IACUC.

Materials

ASA was purchased from Sigma (St. Louis, MO), and ATV was from Pfizer Pharmaceuticals (New York, NY). ELISA kits for 6-keto-PGF1α and COX activity, and arachidonic acid, SC-58125, and SC-560 were from Cayman Chemicals (Ann Arbor, MI).

Treatment

Rats received 3-day pretreatment with ATV (10 mg·kg⁻¹·day⁻¹) suspended in water or water alone, administered by oral gavage once daily. On the fourth day all rats underwent coronary artery ligation for 30 min.

Protocol 1. At 27 min of coronary artery occlusion, rats received intravenous ASA (5, 10, or 20 mg/kg), dissolved in saline or equal volume of saline alone over 2 min (Fig. 1). Rats were euthanized after 4 h of reperfusion (IS protocol) or after 10 min of reperfusion (for enzyme expression and activity assays).

Protocol 2. Rats received intravenous ASA (20 mg/kg) or saline alone either immediately after coronary artery occlusion or at 15 min of reperfusion. An additional group of rats received ATV (10 mg·kg⁻¹·day⁻¹) for 3 days and intravenous saline after coronary artery occlusion. Rats were euthanized after 24 h of reperfusion for IS assessment.

Infarct Size Surgical Protocol

The rat model of myocardial ischemia-reperfusion injury has been described in detail (3, 4, 7, 36). On the fourth day, rats were anesthetized with intraperitoneal injection of ketamine (60 mg/kg) and xylazine (6 mg/kg), intubated, and ventilated (FiO₂ = 30%). The rectal temperature was monitored, and body temperature was maintained between 36.7°C and 37.3°C throughout the experiment. The left carotid artery was cannulated. The chest was opened, and the left coronary artery was encircled with a suture and ligated for 30 min. Isoflurane (1–2.5% titrated to effect) was added after the beginning of ischemia to maintain anesthesia. At 30 min of ischemia, after administration of aspirin or saline, the snare was released and myocardial reperfusion was verified by change in the color of the myocardium. In the IS protocol, subcutaneous 0.1 mg/kg buprenorphine was administered, the chest was closed, and the rats recovered from anesthesia. Four hours (protocol 1) or 24 h (protocol 2) after reperfusion the rats were reanesthetized, the coronary artery was re-ocluded, 1.5 ml of Evan’s blue dye 3% were injected into the right ventricle, and the rats were euthanized while under deep anesthesia. Heart rate and mean blood pressure were noted at baseline (10 min after completion of surgery), immediately before coronary artery occlusion, at 25 min of ischemia, and at 20 min of reperfusion.

The prespecified exclusion criteria were lack of signs of ischemia during coronary artery ligation, lack of signs of reperfusion after release of the snare, prolonged ventricular arrhythmia with hypotension, and area at risk (AR) ≤ 10% of the left ventricular weight.

Myocardial Levels of 6-Keto-PGF₁α and COX Activity Protocol

Ten minutes after reperfusion, the coronary artery was reoccluded, 1.5 ml of Evan’s blue dye 3% were injected into the right ventricle, and the rats were euthanized while under deep anesthesia. The ischemic myocardial zone (not colored by the blue dye) was rapidly dissected and stored at −80° until analysis.

Determination of Area At Risk and Infarct Size

Hearts were excised, and the left ventricle was sliced transversely into six sections. Slices were incubated for 10 min at 37°C in 1% buffered (pH 7.4) 2,3,5-triphenyl-tetrazolium-chloride (TTC), fixed in a 10% formaldehyde, and photographed to identify the AR (uncolored by the blue dye), the IS (unstained by TTC), and the nonischemic zones (colored by blue dye). The AR and IS in each slice were determined by planimetry, converted into percentages of the whole for each slice, and multiplied by the weight of the slice, and the results were summed to obtain the weight of the myocardial AR and IS (3, 4, 7, 36).

6-Keto-PGF₁α and Total COX, COX₁, and COX₂ Activity

Myocardial samples were sectioned into four segments (20 mg each), homogenized in cold phosphate-buffered saline (pH 7.4), and then centrifuged. The supernatants of each sample were collected and divided into four test vials containing 500 µl Hanks’ HEPES solution. The first vial was used for assessing 6-keto-PGF₁α levels. The second vial was used for assessing total COX + PGI₂ synthase integrated activity [50 µM arachidonic acid (AA) were added to bypass a possible limiting effect of cPLA₂]. The third vial contained 50 µM AA and 200 µM of SC-58125 (a specific COX2 inhibitor) and was used for assessing 6-keto-PGF₁α generated by COX1. The fourth vial contained 50 µM AA and 100 µM SC-560 (a specific COX1 inhibitor) and was used for assessing 6-keto-PGF₁α generated by COX2 (3, 7). After 15-min incubation at room temperature, the supernatant in each vial was aspirated and stored at −70°C. The samples (25 µl each) were analyzed for 6-keto-PGF₁α, by using immunoassay assay kits (Cayman Chemicals). We also assessed the peroxidase activity of COX1 in the control and ATV group by using an assay kit (Cayman Chemical), as previously described (36).
Statistical Analysis

Data are presented as means ± SE. The significance level α is 0.05. Body weight, left ventricular weight, the size of the AR and IS, 6-keto-PGF₁₄α, levels, and COX activity were compared using analysis of variance with Sidak 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.

RESULTS

Protocol 1

Infarct size. A total of 49 rats were included in the IS protocol (7–8 in each group). Three rats died during reperfusion: one in the ASA group, one in the ATV + 5 mg ASA, and one in the ATV + 10 mg ASA. There were no other exclusions. Body weight and the size of the AR were comparable among groups (Table 1). IS was significantly smaller in the ATV group than in the control group. ASA alone had no significant effect on IS; however, ASA in a dose-dependent way blunted the protective effect of ATV (Table 1, Fig. 2). IS in the ATV + 10 mg ASA and ATV + 20 mg ASA was not significantly different from that of the controls or ASA alone group; however, it was significantly larger than in the ATV alone group.

Hemodynamics. Two-way repeated measures analysis of variance showed that mean blood pressure was not significantly different among groups (\( P = 0.519 \)), although blood pressure significantly changed in all groups over time (\( P < 0.001 \)). Likewise, overall there were no significant differences among the groups in heart rate (\( P = 0.181 \) for the group effect; \( P < 0.001 \) for the time effect).

Myocardial 6-keto-PGF₁₄α and COX activity. Overall, there were significant differences among groups (\( P < 0.001 \)) in myocardial 6-keto-PGF₁₄α levels (Fig. 3A). ATV significantly increased myocardial 6-keto-PGF₁₄α levels, whereas ASA decreased its levels compared with the control group. ASA in a dose-dependent fashion blunted the ATV induction of 6-keto-PGF₁₄α levels. Blood levels of 6-keto-PGF₁₄α were significantly lower in the ATV + 5 mg ASA, ATV + 10 mg ASA, and ATV + 20 mg ASA groups than in the ATV alone group.

Overall, there were significant differences in total COX activity among the groups (\( P < 0.001 \)) in myocardial 6-keto-PGF₁₄α levels (Fig. 3B). ATV increased total COX activity, whereas ASA inhibited it. Total COX activity was significantly lower in the ATV + 5 mg ASA, ATV + 10 mg ASA, and ATV + 20 mg ASA groups than in the ATV alone group.

Table 1. Body weight, left ventricular weight, area at risk, and infarct size (protocol 1)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ASA (20 mg/kg)</th>
<th>ATV (5 mg/kg)</th>
<th>ATV+ASA (10 mg/kg)</th>
<th>ATV+ASA (20 mg/kg)</th>
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<td>8</td>
<td>8</td>
<td>7</td>
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<td>8</td>
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<tr>
<td>Body weight, g</td>
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<td>251±1</td>
<td>251±1</td>
<td>252±1</td>
<td>252±1</td>
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<tr>
<td>AR, % of LV</td>
<td>54.2±2.9</td>
<td>53.7±1.4</td>
<td>49.5±2.8</td>
<td>56.2±1.3</td>
<td>56.7±1.1</td>
<td>55.7±1.0</td>
</tr>
<tr>
<td>IS, % of LV</td>
<td>16.6±1.1</td>
<td>15.8±1.8</td>
<td>4.9±0.6*</td>
<td>8.9±0.5*</td>
<td>12.6±1.1</td>
<td>13.1±2.0</td>
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</tbody>
</table>

Values are means ± SE; n, number of rats. ASA, aspirin; ATV, atorvastatin; AR, area at risk; LV, left ventricle; IS, infarct size. *\( P = 0.001 \) vs. control.
Nevertheless, IS was significantly smaller in the ATV + ASA at 15 min of reperfusion than in the ASA at reperfusion-alone group (37.6 ± 1.0%; P < 0.001).

**DISCUSSION**

In the present study we demonstrate that intravenous ASA, administered either before reperfusion or at the beginning of ischemia at doses comparable to those used in the clinical setting, blunted the IS-limiting effects of ATV. ASA alone inhibited COX1 activity, without affecting COX2 activity (which was minimal as shown in the control group). However, when given to rats pretreated with ATV, ASA attenuated the induction of COX2 activity by ATV.

Previously, we have shown that ATV (10 mg·kg⁻¹·day⁻¹ for 3 days) augmented the production of 6-keto-PGF₁α via COX1 and COX2 (7). COX1 is a constitutive enzyme, and its expression and activity are not inducible. In the present study we checked the production of 6-keto-PGF₁α, a product of both COX1 and PGI₂ synthase, and the peroxidase activity of COX1 using two separate assays. We have previously shown that ATV augments the expression and activity of PGI₂ synthase (3, 7). When measured by an ELISA kit that assesses the peroxidase activity of COX, we found that ATV (10 mg·kg⁻¹·day⁻¹ for 3 days) does not affect COX1 activity (36). Our current findings are in agreement with the previous studies. We have chosen the current method to study the effects of ASA because ASA is known to acetylate the cyclooxygenase site of COX without a known effect on the peroxidase site (23, 27, 29). Thus the increased production of 6-keto-PGF₁α via COX1 is due to upregulation of PGI₂ synthase and not COX1.

We have shown previously that the same regimen of ATV augments myocardial expression and activity of COX2 in rats not subjected to ischemia (3, 7, 36). ATV augments myocardial production of 6-keto-PGF₁α in rat hearts subjected to 15 min of ischemia (7). ATV activates COX2 by induction of inducible nitric oxide synthase that nitrosylates COX2 (3). Inhibition of COX2 by either valdecoxib (administered orally for 3 days together with ATV) (7) or SC-58125 (administered intrave-
nously just before coronary artery occlusion) (3) abrogates the IS-limiting effect of ATV. In the present study, COX2 activity in the hearts of the control rats subjected to 30 min of coronary artery occlusion and 10 min of reperfusion was mildly elevated compared with the values obtained in nonischemic myocardium in the previous studies (3, 7). ATV caused marked elevation in COX2 activity 10 min after reperfusion. This effect was dose dependently attenuated by ASA. There was a negative correlation between COX2 activity and IS (Fig. 5). This suggests that ASA in a dose-dependent manner blunts the protective effect of ATV by inhibiting COX2.

In our model, myocardial ischemia is induced by mechanical compression of the artery and not by an occlusive thrombus overlying a ruptured intracoronary plaque. Therefore, the potential beneficial effect of ASA on platelet function could not have a significant effect as seen in the clinical setting. ASA alone had no effect on IS. Similar results were reported by Libersan et al. (22) in a dog model with a residual critical coronary stenosis after reperfusion. However, we are showing that in doses comparable to those used in the clinical setting of acute myocardial infarction, ASA attenuated the protective effect of ATV by inhibiting COX2. Shimmura et al. (30) reported that oral ASA at 25 mg/kg, but not 5 or 10 mg/kg, attenuates the protective effect of late ischemic preconditioning against myocardial stunning in the rabbit. Intravenous infusion of ASA at 5 and 25 mg/kg does not negate the effect of late ischemic preconditioning on nuclear factor-κB activation and IS limitation in rabbits (17). In contrast, at 130 mg/kg ASA blocks the protective effect of late preconditioning (17). In a conscious sheep model, intravenous ASA at 20 mg/kg, but not at 1.5 mg/kg or 8 mg/kg, blunts the effect of ischemic preconditioning on myocardial stunning (21). On the other hand, when given intravenously to rats, low doses of ASA (1 and 3 mg/kg), administered 5 min before reperfusion, abolishes morphine-induced IS reduction (14). ASA (50 μg/ml) blunts the antiarrhythmic effect of ischemic preconditioning against reperfusion tachyarrhythmias in the isolated rat heart Langendorff model (2).

One may argue that in the clinical setting of ST elevation myocardial infarction, ASA is usually given during ischemia (i.e., on route to the hospital by the emergency medical services) and not just before reperfusion. In such a case, ASA may acetylate COX1 and COX2 in all perfused tissues, except the ischemic myocardial zone. Therefore, by the time of reperfusion, levels of aspirin in the blood are low and the inhibition of COX2 in the reperfused zone will be low. To answer this question, we added protocol 2a (Fig. 6). We are showing that ASA, administered 30 min before reperfusion, completely blocked the protective effect of ATV, refuting the abovementioned hypothesis.

Most of the “reperfusion injury” occurs within minutes of reperfusion (37). “Postconditioning” reduces IS only when applied within the first few minutes of reperfusion (20, 35). Similarly, the IS-limiting effect of insulin infusion is present when given within the first 15 min of reperfusion (18). Insulin

### Table 2. Body weight, left ventricular weight, area at risk, and infarct size (protocol 2)

<table>
<thead>
<tr>
<th></th>
<th>ASA at Occlusion</th>
<th>ATV + ASA at Occlusion</th>
<th>ATV</th>
<th>ASA at Reperfusion</th>
<th>ATV + ASA at Reperfusion</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
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<td>6</td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>0.671</td>
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<td>Body weight, g</td>
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<td>251 ± 1</td>
<td>252 ± 1</td>
<td>251 ± 1</td>
<td>0.075</td>
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<tr>
<td>AR, % of LV</td>
<td>34.9 ± 2.5</td>
<td>32.1 ± 1.7</td>
<td>31.3 ± 2.3</td>
<td>35.0 ± 1.5</td>
<td>33.0 ± 1.9</td>
<td>&lt;0.001</td>
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<tr>
<td>IS, % of LV</td>
<td>12.4 ± 0.9</td>
<td>10.1 ± 0.3</td>
<td>4.0 ± 0.7</td>
<td>13.2 ± 0.4</td>
<td>6.8 ± 1.0</td>
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![Fig. 5. Correlation between COX2 activity and IS in the various groups.](image1)

![Fig. 6. IS (% of the AR) in rats treated with ATV (10 mg·kg⁻¹·day⁻¹ for 3 days; ATV⁺) or water alone (ATV⁻), and intravenous aspirin (20 mg/kg), administered either immediately after coronary artery occlusion or 15 min after reperfusion.](image2)
infusion after 15 min of reperfusion does not affect IS (18). Therefore, it is not surprising that ASA given at 15 min of reperfusion only partially blocked the protective effect of ATV (Fig. 6).

Currently, chewable ASA at a dose of 160–325 mg is recommended for all patients with acute myocardial infarction (1, 33). For a patient with body weight of 80 kg this translates to 2–4 mg/kg. However, higher doses of up to 500 mg (6–7 mg/kg) are commonly used outside the United States (12). These doses are comparable to those used in our present study.

The important role of ASA in patients with ST elevation myocardial infarction has been established by the ISIS-2 trial (28a); however, at the time when the study was conducted few myocardial infarction has been established by the ISIS-2 trial. It is plausible that in these patients, the beneficial effects of ASA on platelet aggregation may be offset by blunting of the protective effects of statins. It is also plausible that other anti-platelet inhibitors not affecting the COX2 pathway such as clopidogrel (25) or thromboxane A2 receptor inhibitors (16) may be more beneficial especially in patients receiving statins. Similar adverse interaction may also occur in stable ischemic heart disease patients with prolonged use of ASA and statin combination, as some of the pleiotropic effects of statins may be mediated via COX2 products. For example, statins augment development of collaterals (13, 26). In contrast, some have suggested that ASA may reduce the recruitment of collaterals (15, 31). Future clinical studies are needed to assess this potential important adverse interaction between ASA and statins.

REFERENCES


