Atorvastatin-induced cardioprotection is mediated by increasing inducible nitric oxide synthase and consequent S-nitrosylation of cyclooxygenase-2

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Atar, Shaul, Yumei Ye, Yu Lin, Sheldon Y. Freeberg, Shawn P. Nishi, Salvatore Rosanio, Ming-He Huang, Barry F. Uretsky, Jose R. Perez-Polo, and Yochai Birnbaum. Atorvastatin-induced cardioprotection is mediated by increasing inducible nitric oxide synthase and consequent S-nitrosylation of cyclooxygenase-2. Am J Physiol Heart Circ Physiol 290: H1960–H1968, 2006. —We determined the effects of cyclooxygenase-1 (COX-1; SC-560), COX-2 (SC-58125), and inducible nitric oxide synthase (iNOS; 1400W) inhibitors on atorvastatin (ATV)-induced myocardial protection and whether iNOS mediates the ATV-induced increases in COX-2. Sprague-Dawley rats received 10 mg ATV·kg−1·day−1 added to drinking water or water alone for 3 days and received intravenous SC-58125, SC-560, 1400W, or vehicle alone. Anesthesia was induced with ketamine and xylazine and maintained with isoflurane. Fifteen minutes after intravenous injection rats underwent 30-min myocardial ischemia followed by 4-h reperfusion [infarct size (IS) protocol], or the hearts were explanted for biochemical analysis and immunoblotting. Left ventricular weight and area at risk (AR) were comparable among groups. ATV reduced IS to 12.7% (SD 3.1) of AR, a reduction of 64% vs. 35.1% (SD 7.6) in the sham-treated group (P < 0.001). SC-58125 and 1400W attenuated the protective effect without affecting IS in the non-ATV-treated rats. ATV increased calcium-independent NOS (iNOS) [11.9 (SD 0.8) vs. 3.9 (SD 0.1) × 1000 counts/min; P < 0.001] and COX-2 [46.7 (SD 1.1) vs. 6.5 (SD 1.4) pg/ml of 6-keto-PGF1α; P < 0.001] activity. Both SC-58125 and 1400W attenuated this increase. SC-58125 did not affect iNOS activity, whereas 1400W blocked iNOS activity. COX-2 was S-nitrosylated in ATV-treated but not sham-treated rats or rats pretreated with 1400W. COX-2 immunoprecipitated with iNOS but not with endothelial nitric oxide synthase. We conclude that ATV reduced IS by increasing the expression of phosphorylated eNOS, iNOS, cyclo-tosolic PLA2 (cPLA2), COX-2, and PGI2 synthase. Coadministration of valdecoxib, a specific COX-2 inhibitor, for 3 days abrogated the myocardial protective effect of ATV and prevented the increase in myocardial cPLA2, COX-2, and PGI2 synthase expression and activity. Valdecoxib did not affect the phosphorylation of eNOS and the expression of iNOS, suggesting that eNOS and iNOS are probably upstream to the activation of the enzymes responsible for PGI2 production (cPLA2, COX-2, and PGI2 synthase) (4).

In the present study we investigated the effects of acute intravenous administration of specific cyclooxygenase-1 (COX-1), COX-2, and iNOS inhibitors on ATV-induced myocardial protection and PGI2 production. In addition, we assessed whether iNOS inhibition leads to blunting of the ATV-mediated increase in PGI2 production by prevention of the increase in cPLA2, COX-2, and PGI2 synthase activity and expression. Finally, we assessed whether iNOS, induced by ATV, causes S-nitrosylation of COX-2.

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 (NIH Pub. No. 85–23, revised 1996). Experiments were conducted on male Sprague-Dawley rats. The study was approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee.

Materials. 1400W was purchased from Sigma (St Louis, MO), and SC-58125, SC-560, PGI2, NOS activity kit, and ELISA kit for 6-keto-PGF1α were purchased from R&D Systems (Minneapolis, MN).

Since the first description of “ischemic preconditioning” as a potent endogenous form of cardioprotection against ischemic injury (39), there have been multiple studies of the underlying mechanisms that provide myocardial protection. Although numerous triggers, mediators, and effectors have been identified in the various animal models (6, 7, 44, 46), there has been a failure of positive outcomes in clinical trials, with the exception of the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor (statin) trials. Several observational (11, 15, 24, 38, 42) and randomized (16, 40) studies suggest that statins protect the human heart during percutaneous coronary interventions (11, 24, 40), coronary bypass grafting (15), and noncardiac vascular surgery (16, 38, 42). The infarct size (IS)-limiting effect of statins is probably independent of their lipid-lowering properties (3, 4, 29, 30, 37, 45, 50, 52, 53) and may be mediated by enhancement of NO production by endothelial nitric oxide synthase (eNOS) (1, 3, 22, 31, 34–36, 53). However, it has been reported that in the delayed form of ischemic preconditioning the initial activation of eNOS leads to activation of both inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) after a 24-h period. Inhibition of either iNOS or COX-2 blocks the protective effect of ischemic preconditioning. It has been suggested that eNOS activation leads to activation of soluble guanylate cyclase, PKCε, NF-κB, and JAK-signal transducers and activators of transcription pathways, leading to activation of both iNOS and COX-2 (2, 6–9).

We previously showed (4) that atorvastatin (ATV), when administered orally for 3 days at a dose of 10 mg·kg−1·day−1, increases the expression of phosphorylated eNOS, iNOS, cytosolic PLA2 (cPLA2), COX-2, and PGI2 synthase. Coadministration of valdecoxib, a specific COX-2 inhibitor, for 3 days abrogated the myocardial protective effect of ATV and prevented the increase in myocardial cPLA2, COX-2, and PGI2 synthase expression and activity. Valdecoxib did not affect the phosphorylation of eNOS and the expression of iNOS, suggesting that eNOS and iNOS are probably upstream to the activation of the enzymes responsible for PGI2 production (cPLA2, COX-2, and PGI2 synthase) (4).

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6-keto-PGF1α were purchased from Cayman Chemicals (Ann Arbor, MI). Protein A agarose and protease inhibitor were purchased from Sigma. Monoclonal anti-cPLA2 and polyclonal anti-PGL2 synthase antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), polyclonal anti-COX-2 and polyclonal anti-iNOS antibodies from Cayman Chemical, and monoclonal anti-β-actin antibody from Sigma. N-(3-maleimidopropionyl)biocytin (MPB) was purchased from Molecular Probes (Eugene, OR). ImmunoPure horseradish peroxidase-conjugated streptavidin was purchased from Pierce Biotechnology (Rockford, IL).

**Drugs and pretreatment.** Rats received 3-day pretreatment with 10 mg ATV·kg⁻¹·day⁻¹ added to drinking water or water alone. In addition, rats received intravenous 1400W (iNOS inhibitor; 1 mg/kg), SC-58125 (COX-2 inhibitor; 5 mg/kg), SC-560 (COX-1 inhibitor; 2.5 mg/kg), or vehicle alone. Fifteen minutes after intravenous administration of inhibitors or vehicles, rats underwent coronary artery ligation (IS protocol) or hearts were explanted without being subjected to ischemia for enzyme activity determination and for immunoblotting. **IS surgical protocol.** The rat model of myocardial ischemia-reperfusion injury has been described in detail (3, 4, 50). On the fourth day rats were anesthetized with intraperitoneal injection of ketamine (60 mg/kg) and xylazine (6 mg/kg), intubated, and ventilated (inspired O₂ fraction = 30%). 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 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. The heart rate (HR) and mean blood pressure (MBP) were noted at baseline (10 min after completion of surgery), before the injection of the specific inhibitors; just 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 left ventricular weight.

**Determination of AR and IS.** Hearts were excised, and the left ventricle was sliced transversely into six or seven sections. Slices were incubated for 10 min at 37°C in 1% buffered (pH = 7.4) 2,3,5-triphenyltetrazolium chloride (TTC), fixed in 10% formaldehyde, and photographed to identify the AR (uncolored by blue dye), the IS (unstained by TTC), and the nonischemic zones (colored by blue dye). The area of 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, 50).

Table 1. Body weight and ischemic area at risk

<table>
<thead>
<tr>
<th></th>
<th>No Inhibitor</th>
<th>SC-58125</th>
<th>SC-560</th>
<th>1400W</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATV(–)</td>
<td>n = 8</td>
<td>n = 8</td>
<td>n = 8</td>
<td>n = 8</td>
</tr>
<tr>
<td>ATV(+)</td>
<td>n = 8</td>
<td>n = 8</td>
<td>n = 8</td>
<td>n = 8</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>245 (17)</td>
<td>248 (20)</td>
<td>245 (10)</td>
<td>245 (10)</td>
</tr>
<tr>
<td>AR, mg</td>
<td>476 (27)</td>
<td>463 (35)</td>
<td>469 (41)</td>
<td>485 (51)</td>
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<tr>
<td>AR, % of LV</td>
<td>58 (3)</td>
<td>56 (4)</td>
<td>57 (5)</td>
<td>59 (6)</td>
</tr>
<tr>
<td>IS, % of LV</td>
<td>20 (5)</td>
<td>7* (2)</td>
<td>20 (8)</td>
<td>17 (3)</td>
</tr>
</tbody>
</table>

Values are means (SD) for n rats. ATV, atorvastatin; AR, ischemic area of risk; LV, left ventricle; IS, infarct size. *P < 0.005, ATV(+) vs. ATV(–); †P < 0.005, ATV(+) with inhibitor vs. ATV(+) without inhibitor; ‡P = 0.009 vs. ATV(–) without inhibitor.

Table 2. Average heart rate during IS experiment

<table>
<thead>
<tr>
<th></th>
<th>ATV Inhibitor</th>
<th>Baseline</th>
<th>Preocclusion</th>
<th>25-min Occlusion</th>
<th>20-min Reperfusion</th>
</tr>
</thead>
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<tr>
<td>–</td>
<td>No</td>
<td>211 (2)</td>
<td>221 (5)</td>
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<tr>
<td>+</td>
<td>No</td>
<td>221 (8)</td>
<td>229 (8)</td>
<td>244 (6)</td>
<td>232 (10)</td>
</tr>
<tr>
<td>–</td>
<td>SC-58125</td>
<td>211 (3)</td>
<td>226 (2)</td>
<td>241 (3)</td>
<td>223 (3)</td>
</tr>
<tr>
<td>+</td>
<td>SC-58125</td>
<td>212 (3)</td>
<td>233 (5)</td>
<td>246 (2)</td>
<td>226 (2)</td>
</tr>
<tr>
<td>–</td>
<td>SC-560</td>
<td>210 (3)</td>
<td>232 (4)</td>
<td>248 (5)‡‡</td>
<td>224 (2)</td>
</tr>
<tr>
<td>+</td>
<td>SC-560</td>
<td>215 (4)</td>
<td>219 (3)</td>
<td>228 (4)‡‡</td>
<td>218 (3)</td>
</tr>
<tr>
<td>–</td>
<td>1400W</td>
<td>213 (2)</td>
<td>226 (3)</td>
<td>238 (6)</td>
<td>224 (3)</td>
</tr>
<tr>
<td>+</td>
<td>1400W</td>
<td>215 (5)</td>
<td>225 (2)</td>
<td>236 (2)</td>
<td>225 (2)</td>
</tr>
</tbody>
</table>

Values (in beats/min) are mean (SD) heart rate. There was a significant time effect (P < 0.001). There were significant differences among the groups (P < 0.001 for the group effect). Heart rate was significantly higher in the ATV-treated group than in the non-ATV-treated group in rats that did not receive an inhibitor. There were no differences in heart rate between the ATV-treated and non-ATV-treated groups in rats receiving 1400W, SC-58125, and SC-560. Among rats not receiving ATV, 1400W, SC-58125, and SC-560 did not change heart rate compared with rats not receiving an inhibitor. However, among rats treated with ATV, 1400W and SC-560, but not SC-58125, were associated with lower heart rate than the group with ATV alone. *P < 0.05, ATV(+) vs. ATV(–); †P < 0.05, ATV(+) with inhibitor vs. ATV(–) without inhibitor. ‡P < 0.05, ATV(+) with inhibitors vs. ATV(+) without inhibitors.

**NOS activity.** Myocardial samples were homogenized and centrifuged at 100,000 g for 60 min. The supernatant, containing the soluble enzyme iNOS, and the pellet, containing membrane-bound eNOS and neuronal nitric oxide synthase [calcium-dependent NOS (cNOS)] were separated. The pellet was resuspended in homogenization buffer. NOS activity was determined by measuring the conversion of L-[¹⁴C]arginine to L-[¹⁴C]citrulline with a commercial kit. For assessing calcium-dependent NOS (cNOS) activity, CaCl₂ was added to the samples. For assessing calcium-independent NOS (iNOS) activity, CaCl₂ was omitted from the solution. NOS activity was expressed as counts per minute.

**6-keto-PGF1α, COX-2 activity, and PGI2 synthase activity.** Myocardial samples were sectioned into four segments (20 mg each), homogenized in cold PBS (pH 7.4), and centrifuged. The supernatants were collected and stored on ice. The segments were placed into test vials with 500 µl of Hank’s HEPES solution. Fifty micromolar arachidonic acid (AA) was added to the second tube, AA and 200 µM SC-58125 to the third tube, and 150 µM PGL2 to the fourth tube (4). COX-2 activity was considered to be the difference in 6-keto-PGF1α between tubes with and without SC-58125. 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.

**Western immunoblotting.** Determinations of cPLA2, COX-2, and PGI2 synthase expression were performed in samples taken from the left ventricle of rats (6 rats in each group). The hearts were rapidly explanted, rinsed in cold PBS (pH 7.4) containing 0.16 mg/ml heparin to remove red blood cells and clots, frozen in liquid nitrogen, and stored at −70°C.
Tissue samples were homogenized in buffer A [mM: 25 Tris–HCl (pH 7.4), 0.5 EDTA, 0.5 EGTA, 1 phenylmethylsulfonyl fluoride, 1 dithiothreitol, 25 NaF, and 1 Na3VO4, with 1% protease inhibitor] and centrifuged for 15 min at 4°C. The pellets were then incubated on ice in buffer B (buffer A + 1% Triton X-100) for 2 h and centrifuged for 12 min at 4°C. The resulting supernatants were collected as membranous fractions (4).

The expression of the proteins was assessed by standard SDS-PAGE and Western immunoblotting (4, 47). The 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 percentage of the expression in the group that did not receive ATV.

Biotin switch assay. S-nitrosylation of COX-2 was determined with the biotin switch method (27). Myocardial samples from the anterior left ventricular wall of rats pretreated with oral 10 mg ATV-kg−1·day−1 for 3 days (n = 6), rats that received oral ATV for 3 days and intravenous 1400 W 15 min before sampling (n = 3), and rats that did not receive ATV (n = 6) were homogenized with HEN buffer [25 mM HEPES (pH 7.4)-0.1 mM EDTA-0.01 mM nucleotriple] and centrifuged at 16,000 g for 10 min at 40°C. Cold acetone was added to precipitate the proteins. The pellets were resuspended in HEN buffer with 1% SDS, with 20 mM sodium ascorbate added to decompose the SNO bonds. The resulting free thiols in the sample were reacted with 0.05 mM biotinylating agent MPB for 30 min at room temperature. The excess MBP was removed by additional protein precipitation in cold acetone. COX-2 was immunoprecipitated with anti-COX-2 polyclonal antibody. Immunoprecipitates were washed three times with HEN buffer and resuspended in 50 µl of HEN containing Laemmli sample buffer, boiled at 95°C for 5 min, loaded on 10% acrylamide gels, and transferred to nitrocellulose. The biotinylated COX-2 protein was detected with horseradish peroxidase-linked streptavidin. All procedures up to biotinylation were performed in the dark. We verified that the immunoprecipitate contained COX-2 by stripping the membranes with a stripping buffer and blotting them again with anti-COX-2 antibodies.

Coimmunoprecipitation. For coimmunoprecipitation, myocardial homogenates (500 µg) from rats that did not receive ATV (n = 3) and ATV-treated rats (n = 3) were incubated with anti-COX-2 antibodies for 4 h, followed by overnight incubation at 4°C with protein A agarose. The sediment was collected after 5-s centrifugation at 16,000, resuspended in 60 µl of 2X sample buffer, and boiled for 5 min. The agarose beads were collected by centrifugation, and the supernatants were subjected to immunoblotting with anti-iNOS, anti-eNOS, or anti-PGL-synthase antibodies. We verified that the immunoprecipitate contained COX-2 by stripping the membranes with a stripping buffer and blotting them again with anti-COX-2 antibodies.

Statistical analysis. Data are presented as means (SD). The significance level α is 0.05. Body weight and the size of the AR were compared by ANOVA. The differences in IS (as % of AR), enzyme activity, and protein expression were compared by two-way ANOVA looking for the effect of ATV and the different inhibitors [with multiple comparison procedures (Holm–Sidak method)]. We used analysis of covariance to assess the significance of ATV treatment and AR on IS. The differences in HR and MBP were compared with two-way repeated-measures ANOVA. Because there were significant differences in MBP among the groups, we used a linear regression model to assess the effects of AR, MBP, and HR during ischemia and ATV treatment on IS.

RESULTS

IS protocol. Sixty-six rats were included in the protocol (Table 1). None of the rats died or was excluded. Body weight and the size of the AR were comparable among groups.

Table 3. Mean blood pressure during IS experiment

<table>
<thead>
<tr>
<th>ATV</th>
<th>Inhibitor</th>
<th>Baseline</th>
<th>Preocclusion</th>
<th>25-min Occlusion</th>
<th>20-min Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>No</td>
<td>101.9 (2.5)</td>
<td>93.0 (4.4)</td>
<td>84.5 (2.3)</td>
<td>93.2 (5.2)</td>
</tr>
<tr>
<td>+</td>
<td>No</td>
<td>108.6 (11.2)*</td>
<td>99.3 (8.9)*</td>
<td>82.7 (9.6)</td>
<td>91.2 (7.4)</td>
</tr>
<tr>
<td>−</td>
<td>SC-58125</td>
<td>103.9 (2.0)</td>
<td>92.1 (1.0)</td>
<td>82.9 (0.4)</td>
<td>95.2 (1.6)</td>
</tr>
<tr>
<td>+</td>
<td>SC-58125</td>
<td>102.7 (3.3)</td>
<td>91.5 (1.4)†</td>
<td>86.0 (3.1)</td>
<td>97.2 (1.4)</td>
</tr>
<tr>
<td>−</td>
<td>SC-560</td>
<td>103.0 (1.3)</td>
<td>91.7 (1.5)</td>
<td>80.9 (1.4)</td>
<td>98.9 (1.8)</td>
</tr>
<tr>
<td>+</td>
<td>SC-560</td>
<td>106.2 (2.3)</td>
<td>95.2 (1.7)</td>
<td>85.4 (1.8)</td>
<td>97.4 (1.7)</td>
</tr>
<tr>
<td>−</td>
<td>1400W</td>
<td>95.6 (3.4)</td>
<td>85.4 (1.8)‡</td>
<td>77.9 (3.4)</td>
<td>88.6 (2.8)</td>
</tr>
<tr>
<td>+</td>
<td>1400W</td>
<td>105.8 (3.6)*</td>
<td>91.8 (1.3)+†</td>
<td>83.6 (1.2)</td>
<td>93.3 (3.2)</td>
</tr>
</tbody>
</table>

Values (in mmHg) are mean (SD) blood pressure (MBP). There was a significant time effect (P < 0.001). There were significant differences among the groups (P < 0.001 for group effect). MBP was not affected by ATV in rats not receiving an inhibitor. However, among rats that received 1400W, MBP was higher in those pretreated with ATV. Among rats receiving SC-58125 and SC-560, there was no difference in MBP between ATV-treated and non-ATV-treated groups. Among rats not receiving ATV, 1400W, but not SC-58125 and SC-560, was associated with lower MBP than in rats not receiving an inhibitor. Among rats pretreated with ATV, the 3 inhibitors did not significantly affect MBP. *P < 0.05, ATV(+) vs. ATV(−); †P < 0.05, ATV(+) with inhibitor vs. ATV(+) without inhibitor; ‡P < 0.05, ATV(−) with inhibitor vs. ATV(−) without inhibitor.
There were significant differences in HR among the groups (P < 0.001 for the group effect) (Table 2) and for MBP (Table 3). These changes would not favor decreasing IS by hemodynamic effects by ATV; if anything, the changes were in a direction that would bias the results against ATV.

IS in rats not receiving ATV was unaffected by inhibitors SC-58125 (unadjusted P = 0.896), SC-560 (unadjusted P = 0.441), and 1400W (unadjusted P = 0.562) (Figs. 1 and 2). ATV reduced IS by 64% [12.7% (SD 3.1) vs. 35.1% (SD 7.6) in sham-treated group; unadjusted P < 0.001]. SC-58125 and 1400W attenuated the protective effect without affecting IS in the non-ATV treated rats. Both SC-58125 (unadjusted P = 0.141) and 1400W (unadjusted P = 0.221) attenuated the protective effect of ATV. IS in the rats receiving SC-560 was significantly smaller in those pretreated with ATV (unadjusted P = 0.003), and the difference in IS between rats receiving ATV with no inhibitor and those receiving ATV + SC-560 was not significant (unadjusted P = 0.139). Multiple linear regression analysis showed that ATV pretreatment (P = 0.037) and ischemic AR (% of left ventricle; P = 0.025), but not MBP (P = 0.731) or HR (P = 0.302) during ischemia, predicted IS (% of left ventricle).

NOS activity. ATV increased eNOS activity by 2.65-fold (Fig. 3A). SC-58125, SC-560, and 1400W did not affect eNOS activity in ATV- and non-ATV-pretreated rats.

ATV also increased iNOS activity by 3.06-fold (Fig. 3B). SC-58125 and SC-560 had no effect on iNOS activity. In contrast, 1400W blocked iNOS activity in rats not treated with ATV and completely prevented the ATV-mediated increase in iNOS activity.

Myocardial 6-keto-PGF1α, cPLA2 activity, COX-2 activity, and PGI2 synthase activity. ATV increased myocardial 6-keto-PGF1α concentrations (Fig. 4A). Both SC-58125 and 1400W prevented this increase, without affecting 6-keto-PGF1α levels in rats not receiving ATV. In contrast, SC-560 had no effect on 6-keto-PGF1α levels in rats irrespective of ATV treatment.

ATV increased COX-2 activity (Fig. 4B). Whereas SC-560 did not affect COX-2 activity in the ATV-treated and non-ATV-treated groups, 1400W and SC-58125 prevented the ATV-mediated increase in COX-2 activity.

PGI2 synthase activity was increased by ATV in both the group that did not receive an inhibitor and the group that received SC-560 (Fig. 4C). Both SC-58125 and 1400W prevented the ATV effect, without attenuating PGI2 synthase activity in the rats that did not receive ATV.

cPLA2 activity was increased by ATV in all groups (Fig. 4D). None of the inhibitors blunted this increase or affected cPLA2 activity in rats not receiving ATV.

Fig. 2. IS as a function of AR and ATV treatment. A: no inhibitor. B: SC-58125. C: SC-560. D: 1400W. Analysis of covariance revealed that the slopes of the regression lines of the ATV(−) and ATV(+) groups were significantly different in the rats with no inhibitor (P < 0.0001) and the rats that received SC-560 (P < 0.0001). However, among the rats that received SC-58125 (P = 0.15) or 1400W (P = 0.33), the slopes of the regression lines were not statistically significant. LV, left ventricle.
Myocardial expression of cPLA2, COX-2, and PGI2 synthase. ATV increased the expression of cPLA2 (Fig. 5). None of the inhibitors affected cPLA2 expression in the rats not receiving ATV. None of the inhibitors had an effect on the ATV-induced increased cPLA2 expression. ATV increased the expression of COX-2 (Fig. 6). Again, all three inhibitors did not affect COX-2 expression in either rats receiving or rats not receiving ATV. Similarly, ATV increased the expression of PGI2 synthase (Fig. 7). All three inhibitors did not affect PGI2 synthase expression in either ATV-treated or non-ATV-treated rats.

**Biotin switch assay.** The assay results showed that COX-2 was S-nitrosylated in the ATV-treated rats, but not in the rats that did not receive ATV. S-nitrosylation was prevented by intravenous administration of 1400W (Fig. 8A). The sham-treated rats had no expression of COX-2. However, COX-2 was detected in the immunoprecipitate in both the ATV-alone and ATV + 1400W-treated rats (Fig. 8B).

**Coimmunoprecipitation.** Coimmunoprecipitation of COX-2 and iNOS (Fig. 9) showed that the ATV-induced upregulated COX-2 protein was physically associated with iNOS. There was no such apparent COX-2-iNOS association in the rats that did not receive ATV. On the other hand, there was no coimmunoprecipitation of either eNOS or PGI2 synthase with COX-2 (data not shown).

**DISCUSSION**

We have shown that ATV markedly reduced IS by 64% and increased cNOS and iNOS activity as well as expression and activity of cPLA2, COX-2, and PGI2 synthase. Acute administration of a selective COX-2 inhibitor blunted the IS-limiting effect of ATV without affecting cNOS and iNOS activity. Likewise, administration of an iNOS inhibitor at a dose that completely abolished the ATV-induced increase in iNOS activity (Fig. 3B), without affecting cNOS activity (Fig. 3A), abrogated the IS-limiting effect of ATV and blocked the activation of COX-2 and PGI2 synthase (Fig. 4). These findings suggest that ATV-induced myocardial protection is mediated by PGI2 and that iNOS is needed for the activation of both COX-2 and PGI2 synthase. We have demonstrated that ATV causes S-nitrosylation of COX-2. Because iNOS, but not eNOS, coimmunoprecipitated with COX-2, and because 1400W, a selective iNOS inhibitor, prevented S-nitrosylation of COX-2, we conclude that iNOS mediates the S-nitrosylation. Interestingly, the ATV-induced increased cPLA2 activity appears to be independent of iNOS, although it is dependent on COX-2 activity.

**ATV-induced myocardial protection and eNOS.** Statins do not reduce IS in eNOS knockout mice, indicating that eNOS activation is essential for the protective effects of statins (1, 17, 29, 33). In a previous study, concomitant treatment with oral valdecoxb, a selective COX-2 inhibitor, for 3 days abrogated the protective effect of ATV without blunting the activation of eNOS by phosphorylation (4). In the present study, both the selective iNOS inhibitor 1400W and the selective COX-2 inhibitor SC-58125 blunted the protective effect of ATV without affecting cNOS activity. All these data suggest that eNOS activation, although essential for initiating the protective mechanism, is probably upstream to other essential steps such as activation of iNOS and COX-2. These findings are in agreement with the proposed mechanisms of the delayed form of ischemic preconditioning (6).

**ATV-induced myocardial protection and iNOS.** iNOS is essential for mediating the cardioprotective effects of late ischemic preconditioning (2, 6, 19, 48, 56, 57), opioid agonists (20, 28, 41), and sildenafil (13, 43). Both fluvastatin and cerivastatin enhance NO production as well as iNOS mRNA and protein expression by lipopolysaccharide in vascular smooth muscle cells (21, 32). Simvastatin failed to reduce myocardial IS in iNOS knockout mice, indicating that eNOS is essential for mediating the cardioprotective effects of late ischemic preconditioning (6).

**ATV-induced myocardial protection and COX-2.** COX-2 and iNOS (45). In contrast, Bulhak et al. (10) reported that iNOS protein myocardial levels did not increase in pigs receiving rosuvastatin for 5 days and then subjected to ischemia and reperfusion. Three-day pretreatment with ATV induced myocardial expression of iNOS (4). In the present study, we confirmed that a 3-day pretreatment with ATV increased calcium-independent NOS (iNOS) activity. Moreover, we demonstrated that complete iNOS inhibition with 1400W abrogated the IS-limiting effect of ATV without affecting cNOS activity. These results suggest that iNOS is essential for mediating the myocardial protective effects of ATV, as has been shown for simvastatin...
Fig. 4. A: myocardial 6-keto-PGF$_{1\alpha}$ levels. $P < 0.001$ for the effect of ATV; $P < 0.001$ for the effect of the inhibitors. *$P < 0.05$ ATV(+) vs. ATV(-); #$P < 0.05$ for ATV + inhibitor vs. ATV alone. B: myocardial cyclooxygenase-2 (COX-2) activity (generation of 6-keto-PGF$_{1\alpha}$ by COX-2). $P < 0.001$ for the effect of ATV; $P < 0.001$ for the effect of the inhibitors. *$P < 0.05$ for ATV(+) vs. ATV(-); #$P < 0.05$ for ATV + inhibitor vs. ATV alone. C: myocardial PGi2 synthase activity. $P < 0.001$ for the effect of ATV; $P < 0.001$ for the effect of the inhibitors. *$P < 0.05$ for ATV(+) vs. ATV(-). D: cytosolic PLA$_2$ (cPLA$_2$) activity. $P < 0.001$ for the effect of ATV; $P = 0.074$ for the effect of the inhibitors. *$P < 0.05$ for ATV(+) vs. ATV(-).

Fig. 5. A: representative immunoblot of cPLA$_2$. B: densitometric analyses of cPLA$_2$. $P < 0.001$ for the effect of ATV; $P = 0.954$ for the effect of the inhibitors. *$P < 0.05$ for ATV(+) vs. ATV(-). Pos Cont, positive control.

Fig. 6. A: representative immunoblot of COX-2. B: densitometric analyses of COX-2. $P < 0.001$ for the effect of ATV; $P = 0.556$ for the effect of the inhibitors. *$P < 0.05$ for ATV(+) vs. ATV(-).
iNOS and COX-2 interaction. Several studies suggested that iNOS is upstream to COX-2 activation in ischemic preconditioning (48). Administration of iNOS inhibitor 24 h after ischemic preconditioning abrogates the increase in myocardial 6-keto-PGF₁α, whereas administration of COX-2 inhibitors at the same time does not affect iNOS activity (48). Xuan et al. (55) showed that 24 h after an ischemic preconditioning stimulus there is an increase in COX-2 expression in both wild-type and iNOS knockout mice. However, in iNOS knockout mice there is no increase in COX-2 activity, as occurs in wild-type mice, suggesting that iNOS is needed for activation but not for the increased expression of COX-2. In contrast, in COX-2 knockout mice preconditioning augments iNOS expression and activity as it does in wild-type mice (55). Coimmunoprecipitation studies have shown that iNOS interacts with COX-2 but not with COX-1, suggesting a direct physical interaction between iNOS and COX-2 (55). Thus it seems that the augmented expression of iNOS and COX-2 occurs in parallel. However, iNOS is needed to activate COX-2.

Our findings agree with the late ischemic preconditioning model. We found that COX-2 activity was dependent on iNOS activity (Fig. 4), whereas iNOS activity was not affected by COX-2 inhibition (Fig. 3). Moreover, we report for the first time that COX-2 was S-nitrosylated in ATV-treated rats but not
in ATV-treated rats that received 1400W, suggesting that iNOS S-nitrosylates COX-2. S-nitrosylation modifies cysteine residues of many proteins, resulting in reversible posttranslational alterations of protein function analogous to those created by phosphorylation or acetylation (25, 49). Although COX-2 and eNOS are mainly membrane bound whereas iNOS is a cytosolic protein, iNOS, and not eNOS, is causing the S-nitrosylation.

We conclude the following. 1) Pretreatment with 10 mg ATV·kg⁻¹·day⁻¹ markedly reduces myocardial IS. 2) Pretreatment with 10 mg ATV·kg⁻¹·day⁻¹ increases cNOS and iNOS activity and myocardial content of 6-keto-PGF₁α by increasing activity and expression of cPLA₂, COX-2, and PGI₂ synthase. 3) ATV-induced activation of COX-2 is caused by S-nitrosylation, mediated by iNOS. 4) 1400W, a specific iNOS inhibitor, abrogated the myocardial protective effect of ATV, prevented the ATV-induced increase in iNOS activity without affecting cNOS activity, prevented the ATV-induced COX-2 S-nitrosylation, and blocked the increase in COX-2 and PGI₂ synthase activity, without affecting their expression. 5) SC-58125, a specific COX-2 inhibitor, abrogated the myocardial protective effect of ATV and prevented the ATV-induced increase in myocardial content of 6-keto-PGF₁α by blocking the ATV-induced increase in COX-2 and PGI₂ synthase activity, without affecting their expression. 6) SC-560, a specific COX-1 inhibitor, did not negate the IS-limiting effect of ATV, did not affect cNOS and iNOS activity, and did not negate the ATV-induced increase in myocardial content of 6-keto-PGF₁α, COX-2, and PGI₂ synthase activity and expression. 7) cPLA₂ protein expression and activity were increased by ATV. Intravenous administration of 1400W, SC-58125, and SC-560 did not change its expression and activity, suggesting that the mechanisms of activation of cPLA₂ and those of activation of COX-2 and PGI₂ synthase by ATV are different and are independent of iNOS activation.

Together, these data suggest that the IS-limiting effect of ATV shares mechanisms similar to those described for late ischemic preconditioning (9). It is yet to be shown that the myocardial protection by statins other than ATV involves activation of iNOS and COX-2.

REFERENCES


