Translational Sciences

Chronic Treatment With Ticagrelor Limits Myocardial Infarct Size
An Adenosine and Cyclooxygenase-2–Dependent Effect

Manjyot K. Nanhwan, Shukuan Ling, Monica Kodakandla, Sven Nylander, Yumei Ye, Yochai Birnbaum

Objective—In a phase III clinical trial (PLATelet inhibition and patient Outcomes, PLATO), ticagrelor provided better clinical outcomes than clopidogrel in patients with acute coronary syndromes. In addition to P2Y₁₂-receptor antagonism, ticagrelor prevents cell uptake of adenosine and has proven able to augment adenosine effects. Adenosine protects the heart against ischemia–reperfusion injury. We compared the effects of clopidogrel and ticagrelor on myocardial infarct size (IS).

Approach and Results—Rats received oral ticagrelor (0, 75, 150, or 300 mg/kg/d) or clopidogrel (30 or 90 mg/kg/d) for 7 days and underwent 30-minute coronary artery ligation and 24-hour reperfusion. Area at risk was assessed by blue dye and IS by 2,3,5-triphenyl-tetrazolium-chloride. Cyclooxygenase-2 (COX2) enzyme activity was assessed by ELISA and expression by real-time polymerase chain reaction. Mechanism responsible was explored using adenosine-receptor antagonist (CGS15943, an A₁/A₂a antagonist) or cyclooxygenase inhibition by either aspirin (5, 10, or 25 mg/kg) or specific cyclooxygenase-1 (SC560) or COX2 (SC5815) inhibitors. Ticagrelor, dose-dependently, reduced IS, whereas clopidogrel had no effect. Adenosine-receptor antagonist blocked the ticagrelor effect and COX2 inhibition by SC5815, or high-dose aspirin attenuated the IS-limiting effect of ticagrelor, whereas cyclooxygenase-1 inhibition or low-dose aspirin had no effect. Ticagrelor, but not clopidogrel, upregulated COX2 expression and activity. Also this effect was blocked by adenosine-receptor antagonist. Ticagrelor, but not clopidogrel, increased Akt and endothelial nitric oxide synthase phosphorylation.

Conclusions—Ticagrelor, but not clopidogrel, reduces myocardial IS. The protective effect of ticagrelor was dependent on adenosine-receptor activation with downstream upregulation of endothelial nitric oxide synthase and COX2 activity. (Arterioscler Thromb Vasc Biol. 2014;34:2078-2085.)

Key Words: adenosine • aspirin • cyclooxygenase • platelet aggregation inhibitors
Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Ticagrelor Limits Myocardial IS

A total of 8 rats died during surgery (2 in the control, 1 in the ticagrelor 75 mg/kg/d, 2 in the ticagrelor 150 mg/kg/d, and 2 in the clopidogrel 90 mg/kg/d group). Body weight, left ventricular (LV) weight, and the size of the area at risk (AR) were comparable among groups (Table 1). IS, expressed as percentage of the LV (Table 1) or percentage of the AR (Figure 1A) was significantly and dose-dependently reduced in the ticagrelor-treated animals, whereas clopidogrel at 30 and 90 mg/kg/d had no effect. Hemodynamic data are presented in Figure 1A and IB in the online-only Data Supplement.

Plasma levels of ticagrelor 16 hours after the last dose, corresponding to time for coronary artery ligation in the IS experiments, are presented in Figure 1B. Levels were 3.35±0.14, 3.68±0.02, and 6.69±0.07 μM in the ticagrelor 75, 150, and 300 mg/kg/d, respectively.

Both ticagrelor and clopidogrel dose-dependently inhibited platelet aggregation (Figure 1C). The differences in level of inhibition between the clopidogrel 30 mg/kg/d and ticagrelor at 75 and 150 mg/kg/d were not significant. Likewise, the difference in inhibition level between clopidogrel 90 mg/kg/d and ticagrelor 300 or 150 mg/kg/d was not significant, suggesting that both drugs were equally effective in inhibiting platelet aggregation at the doses evaluated with next to complete inhibition achieved at clopidogrel 90 mg/kg/d and ticagrelor 150 mg/kg/d.

Cardioprotective Effect of Ticagrelor Is Depend on Adenosine-Receptor Activation

A total of 5 animals died during surgery (1 in the control, 3 in the CGS15943, and 1 in the ticagrelor+CGS15943 group). Body weight, LV weight, and the size of the AR were comparable among groups (Table 2). Again, IS, expressed as percentage of the LV (Table 2) or percentage of the AR (Figure 2A), was significantly reduced by ticagrelor 300 mg/kg/d relative vehicle-treated animals, whereas clopidogrel 90 mg/kg/d was ineffective. CGS15943 alone (10 mg/kg) or in combination with clopidogrel had no effect on IS, whereas it completely abrogated the IS-limiting effects of ticagrelor (P=0.074 ticagrelor+CGS15943 versus control; P=1.00 versus CGS15943 alone; P=0.001 versus ticagrelor alone). Hemodynamic data are presented in Figure 1C and ID in the online-only Data Supplement.

Cardioprotective Effect of Ticagrelor Is Partly Inhibited by High-Dose But Not Low-Dose Aspirin

Two animals died during surgery (1 in the ticagrelor-aspirin 10 mg/kg/d and 1 in the ticagrelor+aspirin 25 mg/kg/d group). Body weight, LV weight, and the size of the AR were comparable among groups (Table 3). Once more, IS, expressed

### Table 1. Body Weight, LV Weight, the Size of the Ischemic Area at Risk, and Infarct Size in the Control, Ticagrelor-Treated, and Clopidogrel-Treated Groups

<table>
<thead>
<tr>
<th></th>
<th>Control (n=8)</th>
<th>Ticagrelor, 75 mg/kg/d (n=8)</th>
<th>Ticagrelor, 150 mg/kg/d (n=8)</th>
<th>Ticagrelor, 300 mg/kg/d (n=8)</th>
<th>Clopidogrel, 30 mg/kg/d (n=9)</th>
<th>Clopidogrel, 90 mg/kg/d (n=9)</th>
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<tr>
<td>Body weight (g)</td>
<td>254±3</td>
<td>257±3</td>
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<td>256±2</td>
<td>258±2</td>
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</tr>
<tr>
<td>LV weight (g)</td>
<td>1.07±0.01</td>
<td>1.09±0.01</td>
<td>1.08±0.01</td>
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<td>1.08±0.01</td>
<td>1.08±0.01</td>
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<tr>
<td>AR (% of LV)</td>
<td>30.2±1.0</td>
<td>30.0±1.1</td>
<td>29.1±0.6</td>
<td>30.5±1.2</td>
<td>29.2±1.2</td>
<td>29.7±0.6</td>
<td>0.894</td>
</tr>
<tr>
<td>IS (% of LV)</td>
<td>16.1±0.9</td>
<td>12.6±0.3†</td>
<td>10.1±0.4†</td>
<td>6.8±0.5†</td>
<td>14.6±0.4†</td>
<td>15.0±0.7*</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

AR indicates area at risk; IS, infarct size; and LV, left ventricular. *P=0.05 vs ticagrelor 300 mg/kg/d; †P<0.05 vs control.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>ACS</th>
<th>acute coronary syndrome</th>
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<tr>
<td>AR</td>
<td>area at risk</td>
</tr>
<tr>
<td>cNOS</td>
<td>calcium-dependent nitric oxide synthase</td>
</tr>
<tr>
<td>COX2</td>
<td>cyclooxygenase-2</td>
</tr>
<tr>
<td>cPLA2</td>
<td>cytosolic phospholipase A2</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>IS</td>
<td>infarct size</td>
</tr>
<tr>
<td>LV</td>
<td>left ventricular</td>
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<tr>
<td>PLAT0</td>
<td>PLTplatelet inhibition and patient Outcomes</td>
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</table>

and that higher doses of aspirin may attenuate COX2 activity and thereby part of the beneficial effects mediated by ticagrelor.

In this study, we evaluated whether pretreatment with ticagrelor or clopidogrel limits myocardial IS, using an experimental model of transient mechanical coronary artery occlusion that is independent of intraluminal thrombus formation. Next, we studied the signaling pathways mediating this protective effect with special emphasis on the role of adenosine-receptor activation and COX2 activity.

Ticagrelor Increases Myocardial COX2 Activity and 6-Keto-PGF1α and 15-Epi-Lipoxin A4 Levels

COX2 activity was undetectable in the myocardium of the control group. Ticagrelor dose-dependently increased COX2 activity, whereas clopidogrel had no effect (Figure 1D). Ticagrelor and clopidogrel did not affect cyclooxygenase-1 activity (data not shown).

Ticagrelor dose-dependently increased both myocardial levels of 6-keto-PGF1α, the stable metabolite of prostacyclin, and 15-epi-lipoxin A4, an anti-inflammatory eicosanoid produced by COX2, whereas clopidogrel had no effect (Figure 1E and IF).
as percentage of the LV (Table 3) or percentage of the AR (Figure 2B), was significantly reduced by ticagrelor 300 mg/kg/d relative to vehicle-treated animals. Aspirin, 5 mg/kg, did not alter the IS reduction by ticagrelor, but 10 mg/kg aspirin tended to attenuate and 25 mg/kg partly but significantly (P<0.001) attenuated the IS reduction by ticagrelor (Figure 2B).

Cardioprotective Effect of Ticagrelor Is COX2 Dependent

Two animals died during surgery (1 in the control and 1 in the ticagrelor+SC5815 group). Again, body weight, LV weight, and the size of the AR were comparable among groups (Table 4). SC5815 and SC560 alone had no effect on IS (Figure 2C). As in the previous experiments, IS, expressed as percentage of the LV

![Figure 1](http://atvb.ahajournals.org/)

**Figure 1.** A, Infarct size (% of the ischemic area at risk). Number of animals in each group are as in Table 1. There were significant differences among groups (P<0.001). *P<0.002 vs control; †P<0.001 vs ticagrelor (TIC) 300 mg/kg/d. B, Ticagrelor blood levels 16 hours after the last dose (n=5 in each group). There were significant differences among groups (P<0.001). *P<0.001 vs control; †P<0.001 vs ticagrelor 300 mg/kg/d. C, Platelet aggregation, induced by 15 μmol/L ADP in platelet reach plasma. There were 5 animals in the control group and 6 in all other groups. There were significant differences among groups (P<0.001). *P<0.002 vs control; †P<0.004 vs ticagrelor 300 mg/kg/d. D, Myocardial cyclooxygenase-2 activity (n=5/group). Overall, there were significant differences among groups (P<0.001). *P<0.004 vs control; †P<0.001 vs ticagrelor 300 mg/kg/d. E, Myocardial 6-keto-PGF1α levels (n=6/group). Overall, there were significant differences among groups (P<0.001). *P<0.001 vs control; †P<0.004 vs ticagrelor 300 mg/kg/d. F, Myocardial 15-epi-lipoxin A4 levels (n=6/group). Overall, there were significant differences among groups (P<0.001). *P<0.01 vs control; †P<0.002 vs ticagrelor 300 mg/kg/d. CLOP indicates clopidogrel; and COX2, cyclooxygenase-2.

<table>
<thead>
<tr>
<th>Table 2. Body Weight, LV Weight, the Size of The Ischemic Area at Risk, and Infarct Size in the Rats Treated With Vehicle (Control), Ticagrelor, Clopidogrel, CGS15943, and Their Combinations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=8)</td>
</tr>
<tr>
<td>Body weight (g)</td>
</tr>
<tr>
<td>LV weight (g)</td>
</tr>
<tr>
<td>AR (% of LV)</td>
</tr>
<tr>
<td>IS (% of LV)</td>
</tr>
</tbody>
</table>

AR indicates area at risk; IS, infarct size; and LV, left ventricular.

*P<0.05 vs ticagrelor 300 mg/kg/d; †P<0.05 vs control.
the IS-limiting effect of ticagrelor, whereas the cyclooxygenase-1 inhibitor (SC560) did not interfere with the ticagrelor effect, as IS was comparable between the ticagrelor alone and the ticagrelor+SC560 groups ($P=0.939$).

**Myocardial Adenosine Levels**
Ticagrelor 300 mg/kg increased myocardial adenosine levels (Figure 3A). Aspirin (25 mg/kg) and CGS15943 (10 mg/kg) alone had no effect on myocardial adenosine levels and they did not alter the ticagrelor effect.

**Myocardial Calcium-Dependent and Calcium-Independent Nitric Oxide Synthase Activity**
Ticagrelor increased myocardial calcium-dependent nitric oxide synthase (cNOS) activity. Aspirin alone had no effect on cNOS activity and did not block the effect of ticagrelor. On the contrary, CGS15943 completely blocked ticagrelor-induced increase in cNOS activity without affecting cNOS activity when administered alone (Figure 3B). In contrast, none of the drugs affected calcium-independent nitric oxide synthase activity (data not shown).

**Myocardial Cytosolic Phospholipase A$_2$ and COX2 Activity**
Ticagrelor increased cytosolic phospholipase A$_2$ (cPLA$_2$; Figure 3C). Aspirin and CGS15943, administered alone, had no effect on cPLA$_2$ activity. However, both aspirin and CGS15943 blocked ticagrelor-induced upregulation of cPLA$_2$ activity. COX2 activity was undetectable in the control group (Figure 3D) and was significantly increased by ticagrelor. Aspirin and CGS15943, administered alone, had no effect on COX2 activity; but both blocked the augmentation by ticagrelor.

**Immunoblotting and Real-Time Polymerase Chain Reaction**
Ticagrelor increased P-Akt and phosphorylated endothelial nitric oxide synthase (P-eNOS) levels, without affecting total levels of Akt or eNOS levels (Figure 4A–4C). Aspirin alone had no effect on P-Akt or P-eNOS levels and it did not block the upregulation by ticagrelor. CGS15943 alone had no effect on P-Akt and P-eNOS levels; however, it completely reversed the increase induced by ticagrelor. Ticagrelor increased COX2 mRNA levels (Figure 4D). Aspirin alone had no effect on COX2 mRNA levels, and it did not block the ticagrelor effect. CGS15943 alone had no effect on COX2 mRNA levels, but it completely abrogated the ticagrelor effect.

### Table 3. Body Weight, LV Weight, the Size of the Ischemic Area at Risk, and Infarct Size in the Rats Treated With Vehicle (Control) or With Ticagrelor Without and With Aspirin

<table>
<thead>
<tr>
<th></th>
<th>Control (n=6)</th>
<th>Ticagrelor, 300 mg/kg/d (n=7)</th>
<th>Ticagrelor+Aspirin, 5 mg/kg (n=6)</th>
<th>Ticagrelor+Aspirin, 10 mg/kg (n=7)</th>
<th>Ticagrelor+Aspirin, 25 mg/kg (n=8)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>253±2</td>
<td>257±2</td>
<td>262±5</td>
<td>271±6</td>
<td>263±6</td>
<td>0.108</td>
</tr>
<tr>
<td>LV weight (g)</td>
<td>1.06±0.01</td>
<td>1.08±0.01</td>
<td>1.07±0.01</td>
<td>1.08±0.01</td>
<td>1.07±0.01</td>
<td>0.095</td>
</tr>
<tr>
<td>AR (% of LV)</td>
<td>30.5±0.8</td>
<td>30.8±0.9</td>
<td>31.8±0.9</td>
<td>31.2±0.9</td>
<td>32.3±1.2</td>
<td>0.675</td>
</tr>
<tr>
<td>IS (% of LV)</td>
<td>16.6±0.9*</td>
<td>5.1±0.5†</td>
<td>4.7±0.4†</td>
<td>6.5±0.6†</td>
<td>10.1±0.8†</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

AR indicates area at risk; IS, infarct size; and LV, left ventricular.
* $P<0.001$ vs ticagrelor 300 mg/kg/d; † $P<0.001$ vs control.
The main findings of the present study are that ticagrelor protects against ischemia–reperfusion injury and limits IS. In contrast, despite dosed to similar and next to complete P2Y12 inhibition as ticagrelor, clopidogrel had no effect on IS. The IS reduction by ticagrelor was highly reproducible as similar reduction was seen in 4 separate experiments. The ticagrelor protective effect was dependent on adenosine-receptor activation with downstream upregulation of eNOS, cPLA2, and COX2 enzyme activity. Blocking COX2 with either specific COX2 inhibitor or high-dose aspirin abrogated the IS-limiting effects of ticagrelor.

The doses selected for ticagrelor and clopidogrel equally and next to completely inhibited ADP-induced platelet aggregation (Figure 1C), confirming that both drugs were compared at equal P2Y12 inhibition level. Plasma levels of ticagrelor 16 hours after the last dose, at time of coronary ligation, were 3.35±0.14, 3.68±0.02, and 6.69 ± 0.07 μM in the ticagrelor 75, 150, and 300 mg/kg/d, respectively. These values are slightly higher than the Cmin and Cmax levels reported in patients following 4 weeks of treatment with the standard dose of 90 mg twice daily (0.4 μM with a SD of 0.2 and 1.4 μM with a SD of 0.7 μM, respectively). Data generated show that ticagrelor, but not clopidogrel, increased myocardial adenosine levels (Figure 3). This is in line with prior data that have shown that ticagrelor-but not clopidogrel-treated patients with ACS have elevated plasma adenosine levels. The mechanism responsible for ticagrelor adenosine-related effects has recently been validated to be inhibition of the equilibrative nucleoside transporter-1. The same study also showed that the equilibrative nucleoside transporter-1 inhibition is unique to ticagrelor and not shared with the other P2Y12 antagonists. Aspirin did not alter the ticagrelor-induced effects on adenosine levels, suggesting that COX2 upregulation is downstream to adenosine-receptor activation. Adenosine is essential for the myocardial protection by ischemic preconditioning and various pharmacological preconditioning. For instance, statins activate ecto-5′ nucleotidase that converts AMP into adenosine, and the IS-limiting effect of statins is dependent on adenosine-receptor activation.

We found that the IS-limiting effect of ticagrelor was abrogated with CGS15943, an adenosine-receptor antagonist, whereas CGS15943 alone or in combination with clopidogrel had no effect (Figure 2). Moreover, CGS15943 abolished ticagrelor-induced upregulation of eNOS, cPLA2, and COX2 activities (Figure 3), upregulation of Akt and eNOS phosphorylation.

### Table 4. Body Weight, LV Weight, the Size of the Ischemic Area at Risk, and Infarct Size in the Rats Treated With Vehicle (Control), Ticagrelor Alone, SC5815 Alone, SC560 Alone, and Their Combination

<table>
<thead>
<tr>
<th>Condition</th>
<th>Body Weight (g)</th>
<th>LV Weight (g)</th>
<th>AR (% of LV)</th>
<th>IS (% of LV)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=4)</td>
<td>253±3</td>
<td>1.07±0.01</td>
<td>31.1±0.9</td>
<td>31.4±0.9</td>
<td>0.700</td>
</tr>
<tr>
<td>Ticagrelor, 300 mg/kg/d (n=4)</td>
<td>258±2</td>
<td>1.07±0.01</td>
<td>30.1±1.7</td>
<td>31.4±1.1</td>
<td>0.213</td>
</tr>
<tr>
<td>SC5815, 5 mg/kg (n=4)</td>
<td>25±3</td>
<td>1.08±0.01</td>
<td>30.4±0.9</td>
<td>31.4±1.0</td>
<td>0.658</td>
</tr>
<tr>
<td>Ticagrelor+SC5815 (n=4)</td>
<td>25±1</td>
<td>1.08±0.01</td>
<td>31.4±0.9</td>
<td>33.0±1.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SC560, 2.5 mg/kg (n=4)</td>
<td>257±7</td>
<td>1.07±0.01</td>
<td>14.3±1.0</td>
<td>15.1±1.0</td>
<td>6.2±0.4*</td>
</tr>
<tr>
<td>Ticagrelor+SC560 (n=4)</td>
<td>25±6</td>
<td>1.08±0.01</td>
<td>14.3±1.0</td>
<td>15.1±1.0</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

AR indicates area at risk; IS, infarct size; and LV, left ventricular. *P<0.001 vs control.

### Figure 3. A, Myocardial adenosine levels (n=4/group). Overall, there were significant differences among groups (P<0.001). *P<0.005 vs control. B, Myocardial calcium-dependent nitric oxide synthase (cNOS) activity (n=4/group). Overall, there were significant differences among groups (P<0.001). *P<0.013 vs control. C, Myocardial cytosolic phospholipase A2 (cPLA2) activity (n=4/group). Overall, there were significant differences among groups (P<0.001). *P<0.001 vs control. D, Myocardial cyclooxygenase-2 (COX2) activity (n=4/group). Overall, there were significant differences among groups (P<0.007). *P=0.004 vs control. ASA indicates aspirin; CGS, CGS15943; and TIC, ticagrelor.
study that compared the effects of prasugrel with clopidogrel showed that prasugrel reduced the risk of the composite of cardiovascular mortality, myocardial infarction, and stroke in patients with ACS scheduled for percutaneous coronary inter-
vention, irrespective of the aspirin dose. Moreover, such an adverse interaction has not been reported to occur between aspirin and clopidogrel in the Clopidogrel in Unstable angina to prevent Recurrent Events (CURE) study. Here, we offer an alternative explanation. In addition to its antiplatelet effect, ticagrelor via its additional mode of action on equilibrative nucleoside transporter-1, an effect not shared by the thienopyridines, provides protection against ischemia–reperfusion injury.

Urupgulation of COX2, induced by adenosine, is essential for mediating the myocardial IS-limiting effects of delayed ischemic preconditioning. This has also been shown for statins and pioglitazone. Here, we show that ticagrelor, but not clopidogrel, upregulates COX2 mRNA levels and COX2 activity, resulting in increased levels of prostacyclin, as well as 15-epi-lipoxin A4. As the protective effect involves COX2 activation with an increased production of prostaglandins, COX2 inhibition by direct COX2 inhibitors or high doses of aspirin abrogates the IS-limiting effect of ticagrelor. Thus, COX2 inhibition by high-dose aspirin and its impact on the IS-limiting effect of ticagrelor (and potential other eicosanoid-mediated effects) may explain the association between high maintenance dose of aspirin and decrease in relative efficacy of ticagrelor. A recent study has also found that ticagrelor, but not clopidogrel, induced COX2 expression in vitro in primary human aortic endothelial cells stimulated with tumor necrosis factor-α.

In addition to upregulation of COX2, ticagrelor increased cPLA2 activity (Figure 3). This effect was blocked by the adenosine-receptor inhibitor (CGS15943) and aspirin. We have previously shown that atorvastatin increases myocardial cPLA2 expression and activity in the rat. Valdecoxib (a specific COX2 inhibitor) attenuates the effects of statins. Aspirin and sulindac (a nonspecific cyclooxygenase inhibitor) decreases cPLA2 mRNA expression. As cPLA2 is the major enzyme that supplies arachidonic acid to the COX2 for the production of protective prostaglandins, augmentation of cPLA2 activity may contributes to the protective effect of ticagrelor. Indeed, cPLA2 is involved in protecting the heart against ischemia–reperfusion injury.

Thus, both statins and ticagrelor share a similar adenosine-dependent mode of action, as statins can increase the production of adenosine (by activating ecto-5′ nucleotidase) and ticagrelor conserves its extracellular presence by protecting it from its intracellular metabolism. Both mechanism leads to increased adenosine levels providing local enhanced adenosine-receptor activation with downstream activation of Akt, eNOS, and COX2.

The IS-limiting effect of both statins and ticagrelor is abro-
gated by COX2 inhibition with either a specific inhibitor or high-dose aspirin. Thus, an additive, if not synergistic effects, can be hypothesized for ticagrelor and statins. Indeed, the superiority of ticagrelor over clopidogrel in the PLATO trial seems to be reduced in patients not taking lipid-lowering drugs at randomization (P=0.04 for the interaction). Clearly, such an observation is limited, as no information exists about the continued intake of statins. But, if one assume the majority of

Figure 4. A, Samples of immunoblots for P-Akt, total Akt, phospho-
ylated endothelial nitric oxide synthase (P-eNOS), and total eNOS. B, Densitometric analysis of P-Akt levels (n=3/group). C, Densitometric analysis of P-eNOS levels (n=3/group). D, Real-time polymerase chain reaction of cyclooxygenase-2 mRNA lev-
els (n=4/group). Overall, there were significant differences among groups (P<0.001). *P<0.003 vs control. ASA indicates aspirin; CGS, CGS15943; COX2, cyclooxygenase-2; and TIC, ticagrelor.

and upregulation of COX2 mRNA levels (Figure 4). These data confirm that adenosine-receptor activation mediates the upregu-
lation of the prosurvival pathway by ticagrelor.

Both the delayed form of ischemic preconditioning and statins-induced protection are dependent on eNOS activation. We show that ticagrelor, but not clopidogrel, increased cNOS activity (Figure 3) and eNOS phosphorylation at Ser-1177 (Figure 4) via an adenosine-receptor–dependent mechanism. It has previously been reported that adenosine-receptor activation leads to eNOS phosphorylation and that eNOS activation is essential for downstream upregulation of COX2 activity.

The PLATO trial showed that ticagrelor was associated with lower incidence of the composite end point of cardiovascular mortality, myocardial infarction, and stroke than clopidogrel in patients with ACS treated with aspirin. However, a suban-
alysis of the trial data identified a geographic region interaction (P=0.045), suggesting reduced efficacy of ticagrelor versus clop-
idogrel in North American patients. Even if play of chance cannot be excluded, an interaction with the maintenance dose of aspirin and ticagrelor that is linked to the level of P2Y12 inhibition and the potential pro-
the effect of high-dose aspirin through the suppression of prostacyclin. However, even though the study design is different, the TRial to assess Improvement in Therapeutic Outcomes by optimizing platelet inhibition with prasugrel–Thrombolysis In Myocardial Infarction (TRITON-TIMI) 38...
patients that are on statins at randomization continues throughout the study, the data are hypothesis generating.

Wang et al\(^4\) compared the effect of intravenous ticagrelor and clopidogrel, added to intravenous thrombolytic therapy with tissue plasminogen activator in dogs with electrolytic injury-induced intracranial thrombus.\(^4\) In this model, ticagrelor, but not clopidogrel, reduced myocardial IS by \(\approx 60\%\). As both clopidogrel and ticagrelor, like in our study, were dosed to complete inhibition of ex vivo ADP-induced aggregation, they also raised the possibility that the beneficial effect could be mediated by nonplatelet mechanisms, including inhibition of vascular P2\(_Y\), or via adenosine uptake inhibition.\(^4\) Yang et al\(^4\), on the other hand, found that 2-day pretreatment with clopidogrel (15 mg/kg) significantly reduce myocardial IS in rabbits exposed to 30-minute ischemia and 3-hour reperfusion. The protective effect of clopidogrel was abrogated with MRS1754, a selective adenosine A\(_2\B\) receptor blocker, and wortmannin, a PI3 kinase/Akt inhibitor. Intravenous infusion of the short-acting P2\(_Y\), antagonist cangrelor (60 \(\mu\)g/kg bolus 10 minutes before reperfusion followed by 6 \(\mu\)g/kg/min for 3 hours) also reduced IS in their model.\(^4\) The explanation for the difference between the studies concerning the protective effects of clopidogrel is unclear and might be related to the species, the dose, the length of reperfusion (3 versus 24 hour), or a random effect. Importantly, in our study as in the study by Wang et al\(^4\), ticagrelor and clopidogrel were compared at doses providing equally platelet inhibition level. Therefore, the lack of IS-limiting effect of clopidogrel cannot be explained by suboptimal P2\(_Y\), inhibition.

We have tested the effects of 7-day preconditioning with ticagrelor or clopidogrel on myocardial protection; thus, it may be considered to represent a form of “delayed preconditioning.” Currently, we are studying the effects of treatment administered just before reperfusion. This issue should be addressed in further studies. Currently, ticagrelor is indicated for patients after ACSs. The PLATO trial included patients with ST-elevation myocardial infarction and patients with non-ST-elevation ACSs. The duration of therapy in PLATO was 6 to 12 months (median, 277 days) post ACS, and follow-up for outcomes was 1 year.\(^4\) Thus, the PLATO study assesses the outcomes of long-term and not just the acute administration before reperfusion. Most of the clinical events occur 60 days and more after study initiation. Hence, our study imitates a clinical scenario of a patient who develops acute ischemia while being treated with ticagrelor versus clopidogrel for an intermediate or long-term duration.

Conclusions
In addition to its potent P2\(_Y\), antagonism, ticagrelor increases adenosine levels resulting in adenosine-receptor-mediated activation of Akt, eNOS, and COX2. This in turn results in increased production of prostacyclin and 15-epi-lipoxin A\(_3\) and a reduction in IS following ischemia–reperfusion. The protective effect is reversed by adenosine-receptor antagonism but also abrogated with high-dose aspirin, as well as selective COX2 inhibition. This may help explain the mortality benefit seen with ticagrelor and the clinical paradox observed showing reduced efficacy of ticagrelor at higher maintenance doses of aspirin. Further studies are needed to determine the importance of the myocardial protective effects of ticagrelor in the clinical setting.

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Disclosures
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References

Significance
Ticagrelor is an oral platelet P2Y12 receptor antagonist used to prevent atherothrombotic events in patients with acute coronary syndrome. In addition, it increases interstitial adenosine levels. At present, the clinical community is seeking to understand if the additional effect on adenosine levels provides beneficial additional effects beyond other available P2Y12 antagonists. Although our study is preclinical, it addresses 2 relevant clinical issues concerning antplatelet therapy and ischemia–reperfusion injury: (1) ticagrelor, but not clopidogrel, reduces infarct size and this effect is mediated by adenosine-receptor activation; (2) the beneficial effect of ticagrelor is cyclooxygenase-2–dependent and high doses of aspirin attenuate the protective effect. Thus, our study may provide an explanation for the interaction between ticagrelor and aspirin dose that was seen in the pivotal PLATelet inhibition and patient Outcomes (PLATO) trial, which examined the efficacy and safety of ticagrelor vs clopidogrel in patients with acute coronary syndrome.
Chronic Treatment With Ticagrelor Limits Myocardial Infarct Size: An Adenosine and Cyclooxygenase-2–Dependent Effect
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Materials and Methods:

Animal care

This study was approved by The University of Texas Medical Branch IACUC and conducted in accordance with ‘The Guide for the Care and Use of Laboratory Animals’ published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Male Sprague-Dawley rats were used in the study.

Materials

TIC was provided by AstraZeneca and CLOP was purchased from Bristol-Myers Squibb/ Sanofi Pharmaceuticals. Aspirin, CGS15943 and anti-β-actin antibodies were from Sigma (St Louis, MO). ELISA kits for 6-keto-PGF1α and cPLA2 activity, NOS activity kit, arachidonic acid, SC560 and SC58125 was from Cayman Chemicals (Ann Arbor, MI). Immunoassay kit for 15-epi-LXA4 was from Oxford Biomedical Research (Rochester Hills, MI). Anti-AKT and anti-phospho-AKT antibodies were from R&D Systems (Minneapolis, MN). Anti-Ser1177 Phospho-eNOS and anti-eNOS antibodies were from Cell Signaling (Cell Signaling, Beverly, MA).

CGS15943 is a potent A1 and A2A receptor antagonist with moderate affinity for A2B and A3 receptor.1 It is considered to be a nonselective adenosine receptor inhibitor.2

Treatment

Experiment 1: Does TIC limit myocardial IS?

Rats received TIC (0, 75, 150 or 300 mg/kg/d) or CLOP (0, 30 or 90 mg/kg/d) by oral gavage once daily for 7 days. Sixteen hours post last dose on day 8, rats were subjected to 30 min coronary artery ligation and 24h reperfusion. Additional animals were dosed as above and 16h post last dose blood was withdrawn for assessing TIC plasma levels and platelet function or hearts were explanted for immunoblotting and biochemical analysis without being exposed to ischemia-reperfusion.

Experiment 2: Is the protective effect of TIC dependent on adenosine receptor activation?

Rats received vehicle, TIC (300 mg/kg/d) or CLOP (90 mg/kg/d) for 7 days by oral gavage once daily. Fifteen hours post last dose on day 8, CGS15943 (an A2A/A1 adenosine receptor antagonist, 10 mg/kg) or vehicle were administered intraperitoneally. One hour later, rats were subjected to 30 min coronary artery occlusion followed by 24h of reperfusion.

Experiment 3: Is the protective effect of TIC affected by aspirin?

Rats received TIC (300 mg/kg/d) for 7 days without or with aspirin (5, 10, or 25 mg/kg) by oral gavage once daily. Sixteen hours post last dose on day 8, rats were subjected to 30 min coronary artery occlusion followed by 24h of reperfusion.

Experiment 4: Is the protective effect of TIC COX2 dependent?

Rats received TIC (300 mg/kg/d) or vehicle for 7 days by oral gavage once daily. Sixteen hours post last dose, SC58125 (a selective COX2 inhibitor, 5 mg/kg), SC560 (a selective COX1 inhibitor, 2.5 mg/kg) or vehicle were administered intravenously 15 min before coronary artery ligation. Rats were subjected to 30 min coronary artery occlusion followed by 24h of reperfusion.

IS surgical protocol

The rat model of myocardial ischemia-reperfusion injury has been described in detail.3-5 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 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 minutes. Isofluorane (1-2.5% titrated to effect) was added after the beginning of ischemia to maintain anesthesia. After 30 minutes of coronary occlusion, the snare was released and myocardial reperfusion was verified by change in the color of the myocardium. Subcutaneous buprenorphine (0.1 mg/kg) was administered, the chest was closed and the rats were recovered from anesthesia. Twenty-four hours after reperfusion, the rats were re-anesthetized, the coronary artery was reoccluded, 1.5 ml of Evan's blue dye 3% was injected into the right ventricle and the rats euthanized under deep anesthesia. Heart rate and mean blood pressure were noted at baseline (10 minutes after completion of surgery), immediately before coronary artery occlusion, after 25 minutes of ischemia, and following 20 minutes of reperfusion.

The pre-specified 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 ≤ 10% of the LV weight.3-5

**Determination of area at risk (AR) and IS**

Hearts were excised and the left ventricle was sliced transversely into 6 sections. Slices were incubated for 10 minutes at 37°C in 1% buffered (pH=7.4) 2,3,5-triphenyl-tetrazolium-chloride (TTC), fixed in a 10% formaldehyde and photographed in order to identify the AR (unstained by Evan’s blue dye), the IS (unstained by TTC), and the non-ischemic zones (stained by Evan’s blue). The AR and IS area in each slice were determined by planimetry, converted into percentages of the total area, multiplied by the weight of the slice and finally data for all 6 sections were combined to obtain the weight of the myocardial AR and IS 3,5-7.

**ELISA**

Rats were treated as in experiment 1 and hearts were rapidly explanted without being exposed to ischemia-reperfusion protocol. Myocardial samples of the anterior wall of the left ventricle were rinsed in PBS solution (pH 7.4) containing 0.16mg/mL heparin to remove red blood cells and clots, homogenized in cold PBS (pH 7.4) and centrifuged at 10,000 x g for 15 min at 4°C. The supernatant was diluted with water and acidified to pH 3.5 with 1M HCl. The sample was loaded into C-18 Sep-Pak light column (Waters Corporation, Milford, MA) and washed with 1 mL of water followed by 1 mL of petroleum ether. The sample was eluted with 2 mL of methyl formate and evaporated with N2 and the residue was dissolved in extraction buffer. We followed the manufacturer instruction of the immunoassay kits for measuring 6-keto-PGF$_{1\alpha}$ and 15-epi-LXA$_{4\alpha}$.6,8

**COX2 activity** Myocardial samples were sectioned into three 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 500µL Hanks’ HEPES solution. Fifty µM arachidonic acid (AA) were added to the first tube (for overcoming the potential rate limiting effects of cPLA$_2$ that generates AA); AA + 200µM of SC58125 (a specific COX2 inhibitor) to the second tube; and AA + 100µM of SC560 (a specific COX1 inhibitor) to the third tube. After 15-minute incubation at room temperature, the supernatant in each vial was aspirated and stored at -70°C. The samples (25µL each) were analyzed for 6-keto-PGF$_{1\alpha}$.6,8 by an ELISA kit according to the manufacturer instruction. The first tube represents 6-keto-PGF$_{1\alpha}$ generated by both COX1 and COX2. COX2 activity was calculated as 6-Keto-PGF$_{1\alpha}$ levels in the first minus the second tube and COX1 activity as 6-Keto-PGF$_{1\alpha}$ levels in the first minus the third tube.6,8
cPLA$_2$ activity Tissue samples from the anterior left ventricular wall were homogenized in cold phosphate buffer, centrifuged at 10,000 g for 15 min at 4°C. The supernatant was stored on ice. cPLA$_2$ activity was measured using an ELISA kit according to the manufacturer’s instruction.

Nitric Oxide Synthase (NOS) activity Myocardial samples were homogenized in a buffer (25 mM Tris-HCl (pH 7.4); 1 mM EDTA; and 1 mM EGTA), centrifuged at 10,000 X g for 15 min. The supernatant, containing the soluble enzyme iNOS, and the pellet, containing the membrane-bound eNOS and neuronal NOS (nNOS) [calcium dependent NOS (cNOS)] were separated. The pellet was resuspended in homogenization buffer. NOS activity was determined by measuring the conversion of L-[$^{14}$C]-arginine to L-[$^{14}$C]-citrulline using a commercial kit according to the manufacturer’s instructions. For assessing calcium dependent NOS (cNOS) activity CaCl$_2$ was added to the samples. For assessing calcium independent (iNOS) activity, CaCl$_2$ was omitted from the solution. NOS activity was defined as counts per minute (cpm).

Immunoblotting

Myocardial samples from the left ventricle of animals not exposed to ischemia were homogenized in lysis buffer (in mM): 25 Tris·HCl (pH 7.4), 0.5 EDTA, 0.5 EGTA, 1 phenylmethylsulfonyl fluoride, 1 dithiothreitol, 25 NaF, 1 Na$_3$VO$_4$, 1% Triton X-100, 2% SDS and 1% protease inhibitor cocktail. The lysate was centrifuged at 10,000 g for 15 min at 4°C and supernatants were collected. Protein (50µg) was fractionated by SDS-PAGE (4%-20% polyacrylamide gels) and transferred to PVDF membranes (Millipore, Bedford, MA). The membranes were incubated overnight at 4°C with primary antibodies (see "Materials"). Bound antibodies were detected using the chemiluminescent substrate (NEN Life Science Products, Boston, MA). The protein signals were quantified with an image-scanning densitometer, and the strength of each protein signal was normalized to the corresponding β-actin signal. Data are expressed as percent relative the expression in the control group.

Plasma Ticagrelor levels

Blood was collected from rats in experiment 1 from the tail-vein into EDTA anti-coagulated tubes. All blood samples were gently mixed, and immediately placed on ice. Plasma was prepared within 30 minutes of blood sampling by centrifugation at 1500 x g for 10 min at approximately 4°C. The plasma was transferred into tubes stored at or below -20°C within 1 hour of sample collection and plasma concentration of ticargelor was determined by protein precipitation and liquid chromatography mass spectrometry as described previously.

Platelet aggregation

Platelet-rich plasma (PRP) was prepared from blood sampled from rats in experiment 1 by centrifugation of whole blood anticoagulated with citrate dextrose at 800 rpm for 10 minutes at room temperature. Platelet aggregation were recorded after activation with 15 µM ADP using a platelet aggregation profiler (PAP-8; BioData). Data are expressed as the final percent aggregation response after 6 min.

Myocardial adenosine levels

Myocardial samples were immediately shock-frozen with liquid nitrogen. Adenosine levels in myocardial tissue samples were analyzed by high performance liquid chromatography (HPLC) based on the procedure of Wojcik and Neff. Samples were homogenized in 10 volumes of 0.25 M ZnSO$_4$, and protein concentrations were examined by the Lowry assay. After adding ten volumes of 0.25 M Ba(OH)$_2$, the samples were centrifuged at 30,000 x g for 10 minutes. The
supernatants were collected and 5 µL of chloroacetaldehyde was added to 20 µL of supernatant. The tubes were capped, mixed and submerged in a boiling water bath for 10 min. Samples were analyzed by HPLC using a Waters C18 reversed phase 150 mm X 4.6 mm column. The mobile phase was a 50 mM acetate buffer (pH 4.5) and 6.5% aqueous acetonitrile (volume/volume) containing 2 mM sodium octyl sulfonate, 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.

**Real-Time PCR**

RT-PCR was performed by using Applied Biosystems inventoried 20× assay mixes of primers and TaqMan MGB probe (FAM dye labeled) for the target gene COX2 (Mm88242-m1). 18S rRNA (VIC dye-labeled probe) was served as endogenous control. Expression of COX2 relative to the 18s was calculated as the difference between the threshold values of these two genes.
References:


Ticagrelor Supplement

Experiment 1: Does TIC limit myocardial infarct size?- Hemodynamics

There was a significant time effect (p<0.001) and treatment effect (p<0.001) on HR (p=0.001 for the time X treatment interaction) (Figure Ia). The treatment effect was due to differences at HR at baseline. At baseline, HR was faster in the TIC 300 mg/kg/d and CLOP 30 and 90 mg/kg/d than in the control group. The differences between the TIC 75 and 150 mg/kg/d and the control group were not significant. At pre-ooclusion, 25 min of coronary artery occlusion and reperfusion the differences were not significant. Likewise, there was a significant time effect (p<0.001) and treatment effect (p<0.001) on mean blood pressure (MBP), (p=0.001 for the time X treatment interaction). At baseline, MBP was higher in the TIC 300 mg/kg/d and both the CLOP groups compared to the control group (Figure Ib). Yet, at pre-oocclusion, 25 min of coronary artery occlusion and reperfusion the differences were not significant. Thus, TIC did not cause bradycardia or hypotension in the current experiment.

Experiment 2: Is the protective effect of TIC dependent on adenosine receptor activation?- Hemodynamics

There was a significant time effect (p<0.001) and treatment effect (p=0.020) on HR (p<0.001 for the time X treatment interaction) (Figure Ic). At baseline HR was faster in the CGS alone and CGS + TIC groups than in the control group. On the other hand, at pre-occlusion, the only significant difference was that HR was lower in the CLOP alone group than the other groups. During occlusion, HR was lower in the CGS, TIC + CGS and CLOP + CGS groups as compared to the control group. During reperfusion, HR was slower in the CLOP, CGS, TIC + CGS and CLOP + CGS than in the control group. There was no significant effect of treatment on MBP
There was a significant time effect ($p<0.001$ for the treatment X time interaction). At baseline, the differences in MBP were not significant. Before coronary occlusion MBP was higher in the TIC, CGS and TIC + CGS than in the control group (Figure 1d); yet, during ischemia and reperfusion the differences in MBP between each of the treatment groups and the control group were not statistically significant.
Figure I