The Ceiling Effect of Pharmacological Postconditioning with the Phytoestrogen Genistein Is Reversed by the GSK3β Inhibitor SB 216763 [3-(2,4-Dichlorophenyl)-4(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione] through Mitochondrial ATP-Dependent Potassium Channel Opening

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ABSTRACT

In the present study, we investigated the efficacy of pharmacological postconditioning induced by 17β-estradiol and the phytoestrogen, genistein, against myocardial infarction induced by increasing durations of coronary artery occlusion (CAO). Anesthetized rabbits underwent either 20-min (protocol A) or 30-min (protocol B) CAO, followed by 4 h of coronary artery reperfusion (CAR). Before CAR, they randomly received an intravenous injection of either vehicle (control), 100 or 1000 μg/kg genistein (Geni100 and Geni1000, respectively), or 100 μg/kg 17β-estradiol (17β-E100). In protocol A, infarct size was significantly reduced in Geni100 (n = 6), Geni1000 (n = 6), and 17β-E100 (n = 6) versus control (n = 9) (6 ± 2.15 ± 4, and 11 ± 3 versus 35 ± 5%, respectively). In protocol B, none of these drugs reduced infarct size versus control. Western blots demonstrated an increase of Akt phosphorylation in the Geni100 and 17β-E100 hearts submitted to 20-min CAO but not to 30-min CAO. The selective GSK3β inhibitor SB 216763 (0.2 mg/kg [3-(2,4-dichlorophenyl)-4(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione] did not exhibit cardioprotection at this dose, but its administration restored the cardioprotective effect of genistein and 17β-estradiol with 30-min CAO. Administration of 5-hydroxydecanoate (5 mg/kg) abolished the cardioprotective effects of Geni100 and 17β-E100 alone with 20-min CAO and also those observed when combined to SB 216763 with 30-min CAO. Thus, pharmacological postconditioning with genistein and 17β-estradiol is limited by a “ceiling effect of protection” along with a loss of Akt phosphorylation. However, this ceiling effect is reversed by concomitant inhibition of GSK3β by SB 216763 through opening of mitochondrial ATP-dependent potassium channels.
al., 2007), adenosine receptor agonists (Yang et al., 2004a), erythropoietin (Hanlon et al., 2005; Mudalagiri et al., 2008), or isoflurane (Krolikowski et al., 2005; Tessier-Vetzel et al., 2006). In this setting, we previously reported that administration of 17β-estradiol and the phytoestrogen genistein before reperfusion in rabbits also produced pharmacological PCD through activation of the PI3K/Akt pathway and mitochondrial preservation (Tissier et al., 2007).

To date, the cellular mechanism of any investigated cardioprotective strategy has received extensive attention, but only few studies focused on the potential reduction of efficacy with increasing durations of ischemia, i.e., the characterization of a “ceiling of protection” (Murry et al., 1986; Gumina et al., 1999; Tissier et al., 2003). Concerning ischemic PCD, to our knowledge, only one study has reported a ceiling effect. Infarct size was reduced significantly by ischemic PCD after ischemic episodes shorter than 45 min but not after 60 min in conscious rats (Tang et al., 2006). This issue has never been investigated with any reported pharmacological PCD strategy. In accordance, the first goal of the present study was to determine whether a ceiling effect exists with pharmacological PCD induced by 17β-estradiol and the phytoestrogen genistein. It is important to emphasize that ischemic and pharmacological cardioprotective strategies do not always exhibit similar patterns of protection as previously demonstrated (Gumina et al., 1999). Then, we investigated whether the ceiling effect with PCD could be related to differential activation of the PI3K/Akt pathway with increasing durations of ischemia. We next studied whether it was possible to pharmacologically reverse the ceiling effect by concomitant administration of the GSK3β inhibitor SB 216763, i.e., targeting a downstream key step that has been demonstrated to be involved in the signaling pathway of pharmacological PCD (Gross et al., 2004, 2007; Pagel et al., 2006). Finally, we investigated whether the opening of mitochondrial K<sub>ATP</sub> channels was involved in the observed cardioprotection (Gross et al., 2007, 2008).

**Materials and Methods**

The experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996).

**Animal Surgery.** As described previously (Tissier et al., 2003; Couvreur et al., 2006), anesthesia was induced in male New Zealand rabbits (2–3 kg) by zolazepam and tiletamine (both 20–30 mg/kg i.v.). Animals were intubated and mechanically ventilated with 100% oxygen. Anesthesia was maintained by pentobarbital (20–30 mg/kg i.v.). A catheter was positioned in the ear marginal artery for arterial pressure measurement (P23ID strain gauge; Statham Instruments, Oxnard, CA). An external electrocardiogram was also recorded. A left thoracotomy was performed at the fourth intercostal space. The pericardium was opened, and a 3/0 Prolene suture was passed beneath a major branch of the left coronary artery. The ends of the ligature were passed through a short segment of propylene tubing to form a snare. A coronary artery occlusion (CAO) was induced by pulling the snare through the tubing. Ischemia was confirmed by the occurrence of ST segment deviation of the electrocardiogram. Reperfusion was induced by releasing the snare. The chest was then closed in layers.

**Measurement of Risk Area and Infarct Size.** After completion of reperfusion, the animals received heparin and sodium pentobarbital (60 mg/kg i.v.). Potassium chloride was then administered intravenously to induce cardiac arrest, and the hearts were excised. The ascending aorta was cannulated and perfused (120 mm Hg) retrogradely with saline followed by Evans blue (5%) (Sigma-Aldrich, St. Louis, MO) after ligation of the previously occluded artery. The left ventricle was cut into six to eight slices, which were weighed and incubated with 1% triphenyltetrazolium chloride (Sigma-Aldrich) in a pH 7.4 buffer at 37°C. Slices were fixed in 10% formaldehyde and then photographed with a digital camera mounted on a stereomicroscope. Using a computerized planimetric program (Scion Image; Scion Corporation, Frederick, MD), the area at risk and the infarcted zone were quantified. The area at risk was identified as the nonblue region and was expressed as a percentage of the left ventricle weight. Infarcted area was identified as the tissue unstained by triphenyltetrazolium chloride and was expressed as a percentage of the area at risk.

**Experimental Protocol.** The animals were included into five different protocols as illustrated in Figs. 1 and 2. To investigate the ceiling effect, rabbits were subjected either to a 20-min (protocol A) or a 30-min (protocol B) period of CAO. Animals received at random an intravenous injection of vehicle (control), 100 µg/kg 17β-estradiol (17β-E<sub>100</sub>), 100 µg/kg genistein (Geni<sub>100</sub>), or 1000 µg/kg genistein (Geni<sub>1000</sub>) (Sigma-Aldrich). These intravenous injections were performed during the last 5 min of ischemia, before reperfusion. Rabbits underwent 4 h of coronary artery reperfusion (CAR). Heart rate and mean arterial pressure were measured before CAO, during CAO (at 5 and 18 min in protocol A and at 5 and 28 min in protocol B), and at 10-min CAR. To perform myocardial sampling for Western blot analysis, additional rabbits underwent the same protocols, but the animals were sacrificed at 10 min of reperfusion.

In protocol C, to investigate whether inhibition of GSK3β with SB 216763 (0.2 mg/kg i.v.) could prevent the ceiling effect, rabbits received at random 6 min before the onset of CAR SB 216763 alone, SB 216763 plus 17β-E<sub>100</sub>, or SB 216763 plus Geni<sub>100</sub>. Rabbits were submitted to 30-min CAO followed by 4-h CAR. Control animals in protocol B were used as control in protocol C. The dose of SB 216763 was chosen in accordance with the literature. At the dose of 0.2 mg/kg i.v., SB 216763 has no effect on infarct size when administered before reperfusion associated with 30-min CAO in the anesthetized rabbit (Pagel et al., 2006).

In addition, the involvement of mitochondrial K<sub>ATP</sub> channels in the cardioprotective effects observed in protocols A and C was investigated using 5-hydroxydecanoate (5-HD; 5 mg/kg). Additional rabbits were submitted to 20-min CAO (protocol D) in the presence of 5-HD alone, 5-HD + 17β-E<sub>100</sub>, or 5-HD + Geni<sub>100</sub>. Other additional rabbits were submitted to 30-min CAO (protocol E) in the presence of 5-HD alone, 5-HD + SB 216763 + 17β-E<sub>100</sub>, or 5-HD + SB 216763 + Geni<sub>100</sub>. The administration of 5-HD was performed 5 min before CAO.

**Western Blots.** Myocardial samples were homogenized in ice-cold buffer containing 50 mM Tris-HCl, pH 7.4 at 4°C, 150 mM NaCl, 1 mM EDTA, 5 µM protease inhibitor cocktail, 1 mM sodium orthovanadate, 5 mM sodium fluoride, and 1 mM sodium Na<sub>3</sub>B<sub>12</sub>-glycerol phosphate (Sigma-Aldrich). The tissues were scissor minced, washed with ice-cold NaCl, and homogenized using an Ultra-Turrax and a Teflon Potter homogenizer. The homogenates were centrifuged at 40,000g for 30 min at 4°C. The protein concentration was determined by the BCA Protein Assay Kit (Pierce Chemical, Rockford, IL). Proteins were separated on 10% SDS-polyacrylamide gels using 40 µg of proteins per lane. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA). After blocking with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20, membranes were incubated for 1 h at room temperature with the following antibodies: 1:1000 mouse monoclonal anti-phospho-Akt (at Ser473) (Cell Signaling Technology Inc., Danvers, MA), mouse monoclonal anti-Akt 1:1000 (R&D Systems, Minneapolis, MN). The membranes were then washed three times with Tris-buffered saline/Tween 20 for 10 min and subsequently incubated for 1 h with the appropriate secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).
Bands were visualized with chemiluminescence (ECL Western Blotting Substrate; Pierce Chemical), scanned, and quantified in a blinded manner using gel analysis software ImageJ-1.37 (National Institutes of Health, Bethesda, MD).

Statistical Analysis. Data are reported as mean ± S.E.M. Values of left ventricle weights, areas at risk, and infarct sizes were analyzed using a one-way analysis of variance followed by protected least significant difference Fisher test if necessary. Concerning heart rate and mean arterial pressure, the statistical analysis was designed to detect only potential significant changes induced by treatments at each time point measurement (i.e., baseline, CAO 5', CAO 18', CAO 28', CAR 10') using two-way analysis of variance for repeated measures. Significant differences were determined when \( p < 0.05 \).

Results

A total of 124 rabbits were included in this study. The number of animals included in each group is shown in Tables 1 and 2. Concerning Western blots, the experiments were performed on three rabbits in each of the control, 17β-E100, and Geni100 groups submitted either to 20 or 30 min of CAO. Concerning the infarct sizing experiments in protocol A, all rabbits were issued from a previous study from our laboratory (Tissier et al., 2007).

Hemodynamics. As shown in Table 1, values of heart rate and mean arterial pressure were not significantly different among groups in all protocols at baseline, during CAO, and at 10-min CAR.

Area at Risk and Infarct Sizes. As shown in Table 2, left ventricle weights and sizes of the area at risk were similar among groups in each protocol. As illustrated in Fig. 3, left, in animals submitted to 20-min CAO (protocol A), 17β-estradiol and genistein significantly reduced infarct size (69, 83, and 57% in 17β-E100, Geni100, and Geni1000 groups compared with the control group, respectively). However, all these beneficial effects vanished when the duration of ischemia was increased up to 30 min (protocol B, Fig. 3, right), i.e., demonstrating a ceiling effect.

In protocol C, the sole administration of the GSK3β inhibitor SB 216763 at 0.2 mg/kg did not significantly alter infarct size compared with the control (51 ± 6 versus 53 ± 3%, respectively, Fig. 4). It is interesting that the combination of SB 216763 with either 17β-E100 or Geni100 reversed the ceiling effect because these agents significantly reduced infarct size when the hearts were submitted to 30-min CAO (27 ± 8 and 33 ± 5% versus 53 ± 3% in 17β-E100 plus SB
216763 and Geni<sub>100</sub> plus SB 216763 versus control, respectively).

In protocol D (Fig. 5, left), the sole administration of 5-HD did not significantly alter infarct size compared with control (28 ± 5 and 35 ± 5%, respectively). However, 5-HD abolished the cardioprotective effects of Geni<sub>100</sub> and 17β-E<sub>100</sub> after 20 min of CAO (27 ± 5 and 29 ± 3%, respectively). Likewise, in protocol E with 30-min CAO (Fig. 5, right), administration of 5-HD did not significantly alter infarct size compared with control (47 ± 4 versus 53 ± 3%, respectively) but abolished the infarct size-limiting effect of 17β-E<sub>100</sub> + SB 216763 and Geni<sub>100</sub> + SB 216763 (52 ± 8 and 53 ± 5%, respectively).

**Western Blots.** As shown in Fig. 6, Western blot analysis of hearts submitted to 20-min CAO revealed a significant increase in Akt phosphorylation in Geni<sub>100</sub> and 17β-E<sub>100</sub> (both n = 3) compared with control (n = 3). In contrast, Akt phosphorylation in the hearts submitted to 30-min CAO was not increased in Geni<sub>100</sub> and in 17β-E<sub>100</sub> (both n = 3) compared with control (n = 3).

**Discussion**

This study demonstrates for the first time that the cardioprotection afforded by administration of either 17β-estradiol or the phytoestrogen genistein at the onset of reperfusion is limited by a ceiling of protection against myocardial infarction in rabbits. Pharmacological PCD induced by both genistein or 17β-estradiol induced a robust decrease in infarct size after 20-min CAO, but this protection was lost after 30-min CAO. This ceiling of protection was associated with a loss of increase in Akt phosphorylation by both drugs after 30-min CAO. It is interesting that we showed that this ceiling of protection was reversed by inhibiting GSK3β with the concomitant administration of SB 216763 at a dose which has no cardioprotective effect per se. Finally, we observed that these cardioprotective effects observed with 17β-estradiol and genistein alone after 20-min CAO or in combination with SB 216763 after 30-min CAO were abolished when mitochondrial K<sub>ATP</sub> channels were blocked with 5-HD.

To date, the evaluation of any potential cardioprotective
strategy is usually performed using a single duration of CAO. Although this issue is of major importance regarding potential clinical translation, only few studies have investigated whether a ceiling of protection exists, i.e., the efficacy to reduce myocardial infarct size progressively disappears with increasing durations of ischemia (Murry et al., 1986; Gumina et al., 1999; Tissier et al., 2003; Tang et al., 2006). The use of a single CAO duration does not provide exhaustive information about the cardioprotective potency of any investigated drug or maneuver. In accordance and in agreement with our previous report (Tissier et al., 2007), we showed that administration of both genistein and 17β-estradiol induced pharmacological PCD when rabbits were submitted to 20-min CAO. However, these beneficial effects were lost when the duration of CAO was increased up to 30 min. It is unlikely that this loss of cardioprotection was related to insufficient doses of 17β-estradiol and genistein. Regarding 17β-estradiol, its preischemic administration has been shown to be cardioprotective at 10 μg/kg in male rabbits (Hale et al., 1996; Booth et al., 2005), and the same dose administered at the end of 60-min CAO in male dogs has been shown to reduce infarct size (Lee et al., 2004). In the present study, we used a dose of 17β-estradiol that was already 10 times greater to observe cardioprotection with 20-min CAO. Concerning genistein, we did not investigate higher doses than 1000 μg/kg because they are well known to induce tyrosine kinases inhibition (Fryer et al., 1998), which was proposed to alter the protection induced through estrogenic activation (Deodato et al., 1999). It is also well known that prolongation of ischemia is responsible for an increase in infarct size that

### TABLE 1

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<tr>
<th>Groups</th>
<th>Control</th>
<th>17β-E100</th>
<th>Geni100</th>
<th>17β-E100 + Geni100</th>
<th>SB 216763</th>
<th>5-HD</th>
<th>5-HD + 17β-E100</th>
<th>5-HD + Geni100</th>
<th>5-HD + SB 216763 + 17β-E100</th>
<th>5-HD + SB 216763 + Geni100</th>
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<td>LV AAR</td>
<td>268 ± 11</td>
<td>228 ± 12</td>
<td>226 ± 18</td>
<td>252 ± 12</td>
<td>245 ± 13</td>
<td>257 ±26</td>
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<td>258 ± 13</td>
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<td>247 ± 11</td>
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<td>Mean ± S.E.M.</td>
<td>267 ± 11</td>
<td>222 ± 12</td>
<td>219 ± 22</td>
<td>259 ± 10</td>
<td>254 ± 13</td>
<td>257 ± 26</td>
<td>249 ± 13</td>
<td>241 ± 9</td>
<td>263 ± 11</td>
<td>261 ± 14</td>
<td>239 ± 9</td>
<td>233 ± 9</td>
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<tr>
<td>Baseline LV</td>
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<td>224 ± 7</td>
<td>221 ± 17</td>
<td>239 ± 25</td>
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<td>249 ± 17</td>
<td>237 ± 26</td>
<td>263 ± 17</td>
<td>249 ± 14</td>
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### TABLE 2

Left ventricular weight (grams) and area at risk (expressed as percentage of the left ventricle weight)

Values are expressed as mean ± S.E.M.; n, number of rabbits.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>17β-E100</th>
<th>Geni100</th>
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<td>LV AAR</td>
<td>4.4 ± 0.2</td>
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will tend to a maximal value, i.e., approximately 80 to 90% of the area at risk in rabbits (Miura et al., 1989). In the present study, the loss of protection conferred by genistein and 17β-estradiol was observed far from the maximal possible infarct size, with values averaging 50% of the area at risk.

We next investigated whether the ceiling of protection observed with 30-min CAO was related to an inability of the PI3K/Akt pathway to protect the myocardium or whether both 17β-estradiol and genistein were unable to significantly activate the Akt signaling pathway. In the present study, Western blot analysis demonstrated a significant increase in Akt phosphorylation when the hearts were submitted to 20-min CAO. However, when the duration was increased up to 30 min, we did not show any rise in Akt phosphorylation by 17β-estradiol or genistein. This suggests that the loss of protection observed with 30-min CAO results from a loss or modified activation of the Akt pathway. Therefore, it is reasonable to speculate that differential interactions exist among the numerous actors of PCD and those of myocardial injury with varying durations of ischemia. In accordance, we wondered whether other cardioprotective pathways were still activated by 17β-estradiol or genistein despite the loss of protection. In this regard, GSK3β integrates several prosurvival signaling pathways and was shown to be a downstream target of PI3K/Akt pathway (Juhaszova et al., 2004). Moreover, phosphorylation and hence inactivation of GSK3β by estrogens and genistein have been demonstrated previously (Chen et al., 2005; Goodenough et al., 2005; Kajta et al., 2007). Thus, we investigated whether pharmacological PCD with 17β-estradiol and genistein could be recovered after 30-min CAO by potentiating GSK3β inhibition, i.e., with concomitant administration of SB 216763, a selective inhibitor of GSK3β (Gross et al., 2004). At the dose of 0.2 mg/kg, SB 216763 is known to be ineffective at reducing myocardial infarct size when administered before a 30-min CAO in rabbits (Pagel et al., 2006), and our results confirm this finding. In these conditions, the combined administration of SB 216763 with either 17β-estradiol or genistein before reperfusion restored significant reduction in infarct size compared with control. These results suggest that despite the apparent loss of their protective effect, one could speculate that administration of 17β-estradiol or genistein before reperfusion of an episode of 30-min CAO may lead to a partial inhibition of GSK3β. It should be acknowledged that the levels of phosphorylated and total GSK3β were not measured.

We finally investigated the potential role of mitochondrial K$_{ATP}$ channels according to previous reports (Gross et al., 2007, 2008). Administration of 5-hydroxycanoeato abolishes the cardioprotective effects of 17β-estradiol and genistein with 20-min CAO and also those observed when these agents were combined with SB 216763. Thus, our results show that the cardioprotective effects of 17β-estradiol and genistein involve the opening of mitochondrial K$_{ATP}$

**Fig. 3.** Infarct sizes (expressed as percentage of the area at risk) measured in rabbits submitted to 20 min of coronary artery occlusion followed by 4 h of reperfusion (protocol A, left) and 30 min of coronary artery occlusion followed by 4 h of reperfusion (protocol B, right). Open circles, individual infarct sizes; closed circles, mean values ± S.E.M. *p < 0.05 versus control group.

**Fig. 4.** Infarct sizes (expressed as percentage of the area at risk) measured in rabbits submitted to 30 min of coronary artery occlusion followed by 4 h of reperfusion in protocol C. Open circles, individual infarct sizes; closed circles, mean values ± S.E.M. Control animals are those of protocol B. *p < 0.05 versus control group.
channels. In addition, SB 216763 allows reversing the ceiling of cardioprotection observed with these agents, at least in part, through activation of mitochondrial K_ATP channels. Finally, in agreement with previous results concerning ischémic preconditioning (Clarke et al., 2008), one cannot exclude that 17β-estradiol or genistein, in combination or not with SB 216763, will afford cardioprotection through a mechanism mediated by a reduction of oxidative stress.

In conclusion, the cardioprotective effect of pharmacological postconditioning with genistein and 17β-estradiol involves mitochondrial K_ATP channels and is limited by a ceiling effect of protection. This loss of cardioprotection is associated with a loss of Akt phosphorylation capacity with increased durations of ischemia. However, this ceiling effect can be reversed by administration of the GSK3β inhibitor SB 216763 through the opening of mitochondrial K_ATP channels.
Acknowledgments

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