

# Decreased Activity of the Na<sup>+</sup>/H<sup>+</sup> Exchanger by Phosphodiesterase 5A Inhibition Is Attributed to an Increase in Protein Phosphatase Activity

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**Abstract**—The beneficial effect of phosphodiesterase 5A inhibition in ischemia/reperfusion injury and cardiac hypertrophy is well established. Inhibition of the cardiac Na<sup>+</sup>/H<sup>+</sup> exchanger (NHE-1) exerts beneficial effects on these same conditions, and a possible link between these therapeutic strategies was suggested. Experiments were performed in isolated cat cardiomyocytes to gain insight into the intracellular pathway involved in the reduction of NHE-1 activity by phosphodiesterase 5A inhibition. NHE-1 activity was assessed by the rate of intracellular pH recovery from a sustained acidic load in the absence of bicarbonate. Phosphodiesterase 5A inhibition with sildenafil (1 μmol/L) did not affect basal intracellular pH; yet, it did decrease proton efflux (J<sub>H</sub>; in millimoles per liter per minute) after the acidic load (proton efflux: 6.97±0.43 in control versus 3.31±0.58 with sildenafil; *P*<0.05). The blockade of both protein phosphatase 1 and 2A with 100 nmol/L of okadaic acid reverted the sildenafil effect (proton efflux: 6.77±0.82). In contrast, selective inhibition of protein phosphatase 2A (1 nmol/L of okadaic acid or 100 μmol/L of endothall) did not (3.86±1.0 and 2.61±1.2), suggesting that only protein phosphatase 1 was involved in sildenafil-induced NHE-1 inhibition. Moreover, sildenafil prevented the acidosis-induced increase in NHE-1 phosphorylation without affecting activation of the extracellular signal-regulated kinase 1/2-p90<sup>RSK</sup> pathway. Our results suggest that phosphodiesterase 5A inhibition decreases NHE-1 activity, during intracellular pH recovery after an acidic load, by a protein phosphatase 1-dependent reduction in NHE-1 phosphorylation. (*Hypertension*. 2010;56:690-695.)

**Key Words:** signal transduction ■ ion transport ■ phosphatases ■ intracellular acidosis ■ phosphorylation

The cardiac Na<sup>+</sup>/H<sup>+</sup> exchanger (NHE-1) is a 110-kDa glycoprotein expressed at high levels in the myocardium. It extrudes protons concomitantly with Na<sup>+</sup> influx in a 1:1 stoichiometric relationship, rendering the process electroneutral. It has 2 major domains, an NH<sub>2</sub>-terminal membrane transport domain followed by a carboxyl-terminal cytosolic regulatory domain. NHE-1 plays a critical role in a number of cardiovascular disorders including ischemia/reperfusion injury, cardiac remodeling after myocardial infarction, and the development of pathological cardiac hypertrophy.<sup>1-3</sup> Moreover, its specific inhibition has beneficial effects in these circumstances.<sup>4-9</sup> In the last few years, inhibition of the cGMP-catabolizing enzyme, phosphodiesterase 5A (PDE5A), by sildenafil (SIL) has been repeatedly reported to have a beneficial effect in similar pathological conditions.<sup>10-15</sup> By inhibiting PDE5A, SIL raises cytosolic cGMP concentrations leading to protein kinase G (PKG) activation. This kinase has been shown, using pharmacological and gene knockdown approaches, necessary for the cardioprotective action of SIL.<sup>13</sup> However, the downstream targets/mechanism involved in this protective effect remain to be elucidated. Interestingly, we recently demonstrated that the beneficial effect

exerted by PDE5A inhibition, on postmyocardial infarction remodeling in rats, was accompanied by a PKG-dependent inhibition of NHE-1 activity.<sup>16</sup> It was also reported that, in renal mesangial cells, an increase in PKG activity induced NHE-1 inhibition during recovery from an acidic load.<sup>17</sup>

The present study was designed to gain further insight into the cellular mechanism involved in the induction of NHE-1 inhibition by PDE5A inhibition. Therefore, we explored the effect of SIL during intracellular pH (pH<sub>i</sub>) recovery after sustained (5 minutes) intracellular acidosis. Evidence will be presented supporting the hypothesis that cGMP accumulation (because of PDE5A inhibition by SIL) promotes protein phosphatase 1 (PP1) activation, which, in turn, dephosphorylates the regulatory cytosolic tail of the NHE-1, decreasing the exchanger activity.

## Methods

All of the procedures followed during this investigation conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996), and the experimental protocol was approved by the La Plata School of Medicine Animal Welfare Committee. Cats (body weight:

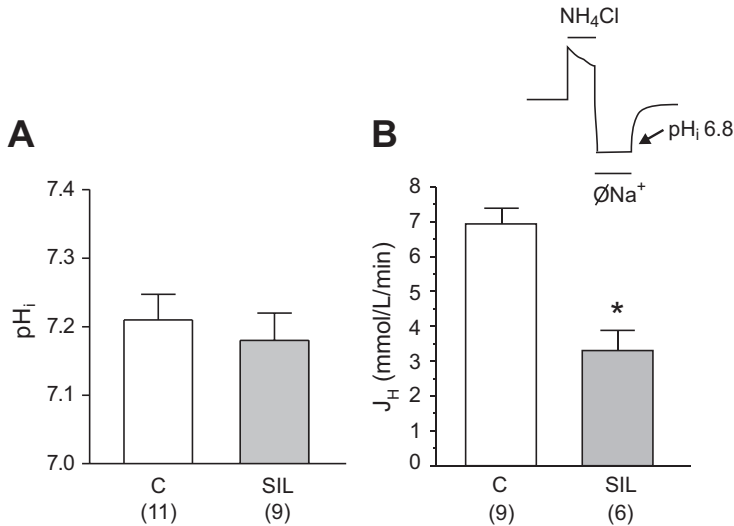
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**Figure 1.** A, NHE-1 activity under PDE5A inhibition. Steady-state pH<sub>i</sub> values were determined in bicarbonate-free medium to solely evaluate NHE-1 activity. The basal pH<sub>i</sub> was not significantly modified by PDE5A inhibition with SIL (1 μmol/L). B, When NHE-1 activity was measured during recovery from a sustained acidic load, it was significantly reduced by PDE5A inhibition with SIL. Insert, Schematic representation of the pH<sub>i</sub> recordings during the experimental protocol followed. Transient exposure to NH<sub>4</sub>Cl was used to induce, by its washout, intracellular acidosis. The duration of intracellular acidosis was extended by an initial washout with Na<sup>+</sup>-free solution, and NHE-1 was reactivated by reintroduction of extracellular Na<sup>+</sup>. J<sub>H</sub> comparison among different groups was done at a common pH<sub>i</sub> of 6.8. The “n” for each experimental group is indicated between brackets. \*P<0.05, Student t test.

3 to 4 kg) were anesthetized by IP injection of sodium pentobarbital (35 mg/kg of body weight), and hearts were rapidly excised when plane 3 of phase III of anesthesia was reached.

For a detail description of the methods, please see the online Data Supplement at <http://hyper.ahajournals.org>.

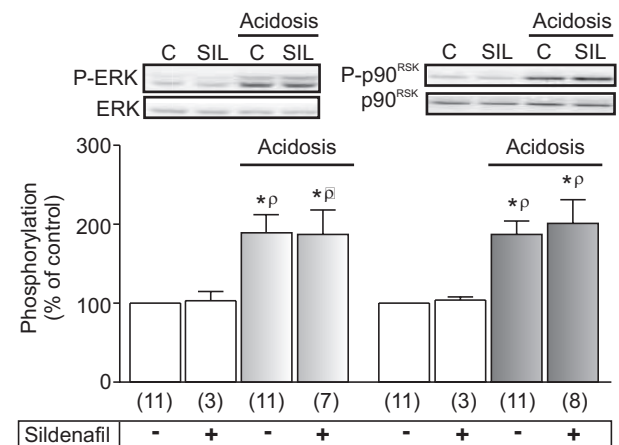
**Results**

NHE-1 activity was assessed by the rate of pH<sub>i</sub> recovery from a sustained acidic load in the absence of bicarbonate. Because NHE-1 activity is regulated by intracellular H<sup>+</sup> concentration, proton efflux (J<sub>H</sub>) comparison among different groups was done at a common pH<sub>i</sub> of 6.8. PDE5A inhibition with SIL did not affect basal pH<sub>i</sub> (Figure 1A); yet, it did significantly decrease the rate of pH<sub>i</sub> recovery after a sustained acidic load (Figure 1B).

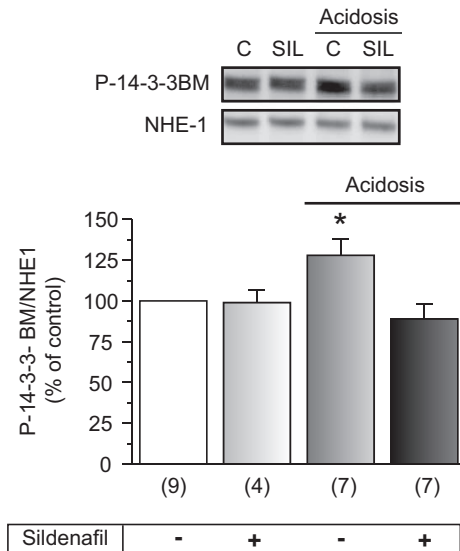
It is well known that NHE-1 activity correlates well with its level of phosphorylation. Considering that sustained intracellular acidosis activates the extracellular signal-regulated kinase (ERK) 1/2-p90<sup>RSK</sup> cascade, leading to phosphorylation of the cytosolic tail of the exchanger,<sup>18</sup> we explored the phosphorylation state of these kinases after acidosis not only in control conditions but also in the presence of SIL. Sustained intracellular acidosis significantly increased ERK1/2 and p90<sup>RSK</sup> phosphorylation, and PDE5A inhibition with was not able to prevent this increase. No effect of SIL on ERK1/2-p90<sup>RSK</sup> basal phosphorylation was evidenced (Figure 2) The fact that SIL suppressed acidosis-induced NHE-1 activity without inhibiting the kinase pathway that underlies the enhanced function of the exchanger suggested to us that a different mechanism was involved in this effect. To get further insight into it, we decided to explore NHE-1 phosphorylation by a phosphospecific antibody, which recognizes the phospho-Ser703 in the 14-3-3 protein binding motif of the carboxyl tail of the NHE-1. This site has been shown to be the target for p90<sup>RSK</sup>.<sup>19,20</sup> As expected, the acidosis-induced activation of the ERK1/2-p90<sup>RSK</sup> pathway increased NHE-1 phosphorylation at Ser703. Interestingly, PDE5A inhibition completely blunted this effect on the exchanger’s cytosolic regulatory domain; however, SIL has no effect on Ser703-NHE-1 phosphorylation under control conditions (Figure 3). Because we found that ERK1/2 and p90<sup>RSK</sup> phosphorylation

was not altered by SIL, we speculated that the effect of PDE5A inhibition on NHE-1 phosphorylation might be because of activation of phosphatases. To explore this hypothesis, we analyzed the SIL effect on NHE-1 activity (J<sub>H</sub>) in the presence of the phosphatase inhibitor, okadaic acid. As shown in Figure 4, 100 nmol/L of okadaic acid, a concentration that inhibits both PP1 and PP2A activity, completely prevented the inhibitory effect of SIL on NHE-1. However, when the concentration of okadaic acid was lowered to 1 nmol/L to make it selective for PP2A,<sup>21,22</sup> no attenuation of the SIL effect was observed. A similar result was found when endothall, another phosphatase inhibitor that has been reported to exhibit greater selectivity for PP2A,<sup>23</sup> was assayed. These results suggested that PP1, but not PP2A, was directly involved in the inhibitory effect on NHE-1 by PDE5A inhibition.

To elucidate whether PP1 directly dephosphorylates the cytosolic tail of the NHE-1, we determined the phosphoryla-



**Figure 2.** Acidosis-induced ERK1/2-p90<sup>RSK</sup> activation: effect of PDE5A inhibition. ERK1/2 and p90<sup>RSK</sup> activation was determined by immunoblot with antibodies specific to the phosphorylated form of each kinase. Acidosis induced a significant increase in both, an effect that was not prevented when acidosis occurred in the presence of SIL. No differences in total ERK-2 and p90<sup>RSK</sup> were observed between groups. The “n” for each experimental group is indicated between brackets. \*P<0.05 vs all other groups, ANOVA.



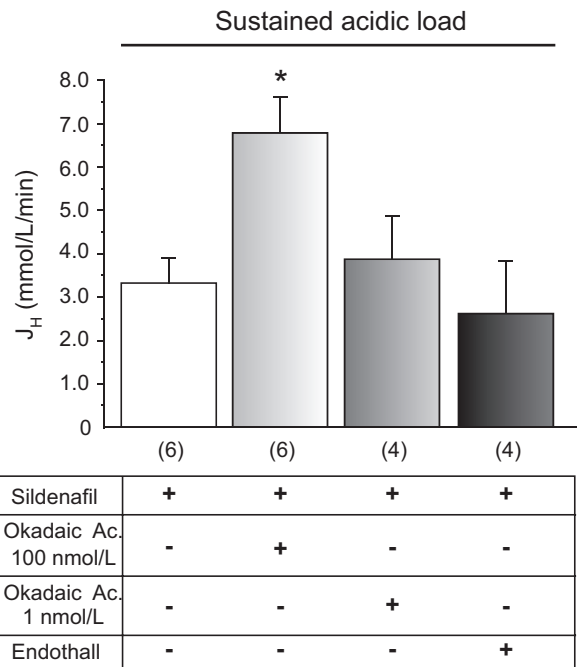
**Figure 3.** Acidosis-induced NHE-1 phosphorylation: effect of PDE5A inhibition. The acidosis-induced increase in ERK1/2-p90<sup>RSK</sup> phosphorylation was accompanied by an increase in NHE-1 phosphorylation at Ser703, estimated by a specific antibody against the P-14-3-3 binding motif (BM). This effect was prevented by PDE5A inhibition (SIL, 1  $\mu$ mol/L). SIL did not affect Ser703-NHE-1 phosphorylation under control conditions (in the absence of sustained acidosis). The “n” for each experimental group is indicated between brackets. \**P*<0.05 vs all other groups, ANOVA.

tion state of the exchanger at Ser703 in the presence of SIL in combination with the protein phosphatase inhibitor okadaic acid. When the highest concentration of okadaic acid (100 nmol/L; both PP1 and PP2A are inhibited) was assayed in combination with SIL, no reduction in acidosis-induced NHE-1 phosphorylation was observed. However, when okadaic acid was assayed at a lower concentration (1 nmol/L; only PP2A is inhibited) SIL effect was not prevented, confirming the involvement of PP1 in the inhibitory action of SIL on NHE-1 activity. Protein phosphatase inhibition with 100 nmol/L of okadaic acid did not significantly alter acidosis-induced Ser703-NHE-1 phosphorylation (Figure 5).

### Discussion

The original finding of the present study is that PDE5A inhibition decreases NHE-1 phosphorylation through the activation of a protein phosphatase (PP1) without interfering with the ERK-p90<sup>RSK</sup> pathway. A schematic representation is shown in Figure 6.

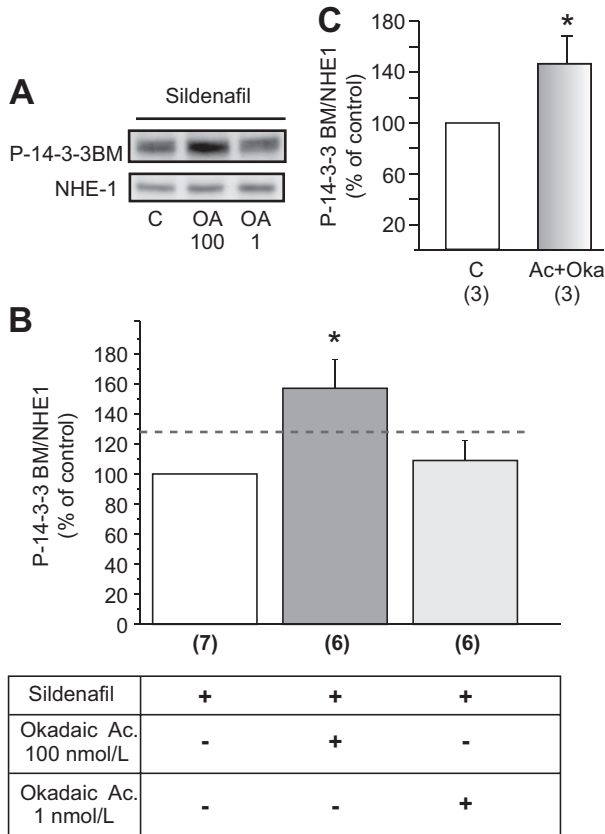
The NHE-1 activity is regulated primarily by intracellular H<sup>+</sup> concentration. It is low at steady-state physiological conditions but increases markedly in response to intracellular acidosis through the interaction of H<sup>+</sup> with an allosteric modifier site within the transport domain.<sup>24</sup> However, additional regulation of NHE-1 activity occurs in response to stretch, altered cell volume, and several neurohumoral factors by posttranslational modification of the carboxyl-terminal cytosolic regulatory tail that modifies the affinity of the allosteric site for H<sup>+</sup>.<sup>25–27</sup> Not only the extent but also the duration of intracellular acidosis regulates NHE-1 activity. Extending the duration of intracellular acidosis has a stimu-



**Figure 4.** SIL effect on NHE-1 activity under protein phosphatase inhibition. The inhibitory effect of SIL on NHE-1 activity ( $J_H$ ) was explored in the presence of the phosphatase inhibitor okadaic acid during the recovery from sustained acidic loads. NHE-1 activity was significantly increased when PP1 activity was inhibited by 100 nmol/L of okadaic acid (OAc 100). On the contrary, selective inhibition of PP2A (1 nmol/L of okadaic acid [OAc 1]; or 100  $\mu$ mol/L of endothall) did not prevent SIL effect on NHE-1 activity. The “n” for each experimental group is indicated between brackets. \**P*<0.05 vs all other groups, ANOVA.

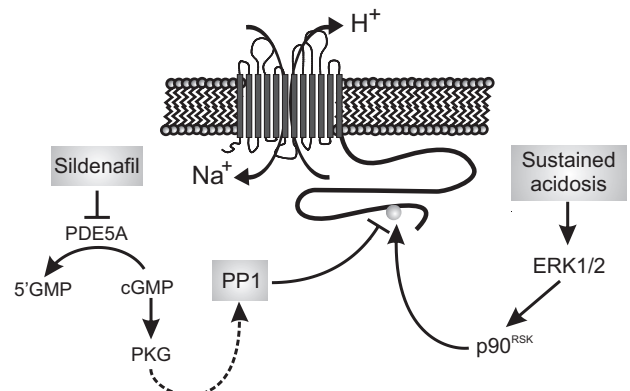
latory effect on NHE-1 activity that depends on the activation of the ERK pathway.<sup>18,28,29</sup> This kinase cascade has been shown to phosphorylate the regulatory domain of the exchanger, increasing its activity.<sup>29–32</sup> In this context, the present study provides insight into a novel regulatory mechanism of NHE-1 activity. We showed that PDE5A inhibition completely blunted the acidosis-induced increase in Ser703 NHE-1 phosphorylation and significantly reduced  $J_H$  after a sustained acidic load (by  $\approx$ 50%). The rationale for studying NHE-1 activity after sustained instead of acute intracellular acidosis was that the former better resembles physiopathologic conditions.

Because the level of phosphorylation of a protein depends on the balance between kinase and phosphatase activity, and SIL significantly decreased NHE-1 phosphorylation at Ser703 without interfering with ERK/p90<sup>RSK</sup> activation, we speculated that PKG activation promoted an increase in phosphatase activity responsible for NHE-1 dephosphorylation. This hypothesis was confirmed by determining  $J_H$  and NHE-1 phosphorylation in the presence of SIL plus phosphatase inhibitors. We found the effect of SIL on the NHE-1 was completely reverted by inhibiting PP1, whereas PP2A appeared not to be involved under our experimental conditions and in agreement with a previous report.<sup>33</sup> We did not detect any effect of PDE5A inhibition on ERK-p90<sup>RSK</sup> phosphorylation, contrary to the finding of Kukreja and colleagues.<sup>13,34</sup> The reason for this difference is not apparent to us at present.



**Figure 5.** NHE-1 phosphorylation under protein phosphatase inhibition. PDE5A inhibition with SIL decreased acidosis-induced NHE-1 phosphorylation at Ser703, estimated by a specific antibody against P-14-3-3 binding motif (BM). This effect was prevented when both PP1 and PP2A were inhibited (100 nmol/L of okadaic acid). However, when only PP2A was inhibited (1 nmol/L of okadaic acid), SIL effect on NHE-1 phosphorylation was not prevented, suggesting that only PP1 was the culprit for NHE-1 dephosphorylation under these experimental conditions. The dash line indicates the value of NHE-1 Ser703 phosphorylation induced by acidosis in the absence of SIL. \**P*<0.05 vs all other groups; ANOVA A: representative blots, top: P-14-3-3 binding motif signal in immunoprecipitated NHE-1 samples; bottom: NHE-1 signal of the same samples, as loading control. C, Sustained acidosis in the presence of 100 nmol/L of okadaic acid (Ac+Oka 100) induced a significant increase in NHE-1 phosphorylation at Ser703 compared with control (C) that was of a similar magnitude to that induced by acidosis itself. \**P*<0.05 vs control, *t* test. The “n” for each experimental group is indicated between brackets.

Intracellular Na<sup>+</sup> accumulation is a key determinant of cardiovascular injury in several pathological conditions, such as ischemic/reperfusion injury and heart failure, and ≈50% of Na<sup>+</sup> entry to the myocytes is through the NHE-1.<sup>35</sup> Therefore, SIL-induced NHE-1 inhibition may explain, at least partially, the cardioprotective effect of this compound. Some other cellular targets and mechanism for PDE5A-inhibition consequences have been anticipated recently. In a mouse model of cardiac pressure overload, PKG-dependent phosphorylation of the regulator of G protein signaling 2 was proposed to mediate the beneficial effect of SIL.<sup>15</sup> Moreover, investigators from this same group have reported lately another novel mechanism underlying the suppression of pathological cardiac hypertrophy by PDE5A inhibitors.<sup>36</sup>



**Figure 6.** Schematic representation of the hypothesis tested and results obtained. Sustained intracellular acidosis has a stimulatory effect on NHE-1 activity that depends on the activation of the ERK1/2-p90<sup>RSK</sup> pathway that phosphorylates the regulatory domain of the exchanger.<sup>18,28–32</sup> PDE5A promotes cGMP accumulation and PKG activation, which, in turn, and by a yet-unknown mechanism, favors PP1 activity, which will dephosphorylate the regulatory cytosolic tail of the NHE-1, decreasing its activity.

They showed that transient receptor potential canonical channels were negatively modulated by PKG-dependent phosphorylation.<sup>36</sup> These stretch sensitive nonselective cationic channels can be responsible for increases in Na<sup>+</sup> and/or Ca<sup>2+</sup> influx mimicking NHE-1 activation. Whether SIL-favored PKG phosphorylation of a transient receptor potential canonical channel is involved in our results was not explored by us but deserves to be considered.

We think the findings of the present work are potentially relevant for the in vivo treatment of several cardiovascular pathologies. Recently, an interesting article by Pokreisz et al<sup>37</sup> reported that PDE5A expression was markedly greater in the left ventricle of patients with dilated and ischemic cardiomyopathy than in unused donor left ventricular tissues. These same investigators found in transgenic mice with PDE5A cardiomyocyte-specific overexpression that this does not affect baseline cardiac function but predisposes mice to adverse left ventricular remodeling after myocardial infarction. This is in line with our previous results in which PDE5A inhibition after coronary artery ligation significantly ameliorated postmyocardial remodeling and left ventricular dysfunction in rats.<sup>16</sup> It is important to note that NHE-1 inhibition through SIL seems not to influence cardiac function and growth under basal conditions, probably limiting by this way potential undesired effects. Based on our previous and present findings, as well as those from others,<sup>13,15,37,38</sup> PDE5A inhibition seems to emerge as an important therapeutic target.

A possible limitation of our study is the reliance on pharmacological inhibitors to analyze the mechanisms involved in PDE5A inhibition-induced consequences. Although at the concentrations used herein these pharmacological compounds have been widely used and probed to be valid tools, the possibility of undesired nonspecific effects of some of them cannot be completely ruled out. On the other hand, we have preliminary results of similar experiments performed in rat papillary muscles in which SIL induces NHE-1 dephosphorylation and inhibition consistently with the results of this



article; however, in that case, PP2A seems to be involved. Whether species differences could be the culprit for this discrepancy is not apparent to us at present.

## Perspectives

NHE-1 inhibition is a powerful therapeutic tool in many cardiovascular pathologies, namely ischemia/reperfusion injury and cardiac hypertrophy/remodeling. Recently, the inhibition of PDE5A has emerged as a promising novel strategy for almost these same cardiac disorders.<sup>13,14,38</sup> SIL is the most widely experimentally and clinically used PDE5A inhibitor. It was the first oral medicine approved for treating erectile dysfunction, and it has another clinically approved use for treatment of pulmonary arterial hypertension. In a recent study we reported a possible link between both therapeutic strategies: chronic treatment with SIL improved postmyocardial infarction remodeling and function through PKG-dependent inhibition of the NHE-1.<sup>16</sup> However, other cellular targets have been also proposed to underlie the cardioprotective effect of SIL.<sup>15,36</sup> In the present study we provide insight into the intracellular pathway involved in the NHE-1-inhibitory effect of SIL, supporting a critical role of PP1 in NHE-1 dephosphorylation and inhibition. Additional research will be necessary to completely elucidate the intracellular mechanisms involved in PDE5A inhibition cardiovascular beneficial effects and their relative importance. On the other hand, pharmacological regulation of protein phosphatase activity emerges as an option in the treatment of cardiac diseases.

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## Disclosures

None.

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