Abstract—The cardioprotection by ischemic preconditioning is lost in aged wild-type and in STAT3 (signal transducer and activator of transcription 3)-deficient mice. The aim of the present study was to analyze whether or not ischemic postconditioning (iPoco) was effective in aged mice hearts and whether iPoco was dependent on STAT3. Young (3 months) and aged (>13 months) C57Bl6/J mice underwent 30 minutes of ischemia and 2 hours of reperfusion without or with iPoco (3 cycles of 10 seconds of ischemia/10 seconds reperfusion [3×10] or 5 cycles of 5 seconds of ischemia/5 seconds of reperfusion [5×5] at the beginning of reperfusion). In young mice, both iPoco×10 and iPoco5×5 reduced infarct size (IS), whereas in aged mice, only iPoco5×5 was effective in reducing IS. In young mice, iPoco3×10 increased the phosphorylated over total STAT3 (phosphorylated STAT3/STAT3) ratio at 10 minutes of reperfusion in the postconditioned anterior wall compared with the control posterior wall. In aged mice hearts, total STAT3 and phosphorylated STAT3/STAT3 in the anterior wall at reperfusion were reduced compared with young mice hearts. In young mice hearts subjected to iPoco×10 but pretreated with the JAK-2 inhibitor AG-490, phosphorylated STAT3/STAT3 was reduced in the anterior wall compared with untreated young mice hearts, and IS reduction by iPoco3×10 was abolished. Furthermore, in young mice with a cardiomyocyte-restricted deletion of STAT3, iPoco3×10 failed to reduce IS, whereas iPoco5×5 reduced IS. Thus, cardioprotection by iPoco is dependent on the postconditioning protocol in aged and STAT3-deficient hearts. The reduced levels of STAT3 with increasing age may contribute to the age-related loss of iPoco. (Circ Res. 2008;102:0-0.)

Key Words: signal transducer and activator of transcription 3 ■ ischemic postconditioning ■ aging

Transient periods of ischemia/reperfusion reduce the irreversible tissue injury by a subsequent prolonged episode of ischemia/reperfusion, a phenomenon known as ischemic preconditioning (IP). Previous studies have demonstrated that IP is not effective in C57Bl6/J mice older than 13 months.1 In young mice hearts subjected to iPoco×10 but pretreated with the JAK-2 inhibitor AG-490, phosphorylated STAT3/STAT3 was reduced in the anterior wall compared with untreated young mice hearts, and IS reduction by iPoco3×10 was abolished. Furthermore, in young mice with a cardiomyocyte-restricted deletion of STAT3, iPoco3×10 failed to reduce IS, whereas iPoco5×5 reduced IS. Thus, cardioprotection by iPoco is dependent on the presence and phosphorylation of STAT3.

Materials and Methods

The present study was performed with approval from the Bioethical Committee of the district of Düsseldorf, Germany, and conformed to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996). C57Bl6/J mice of different ages (young, <3 months; aged, >13 months) were purchased from Charles River Laboratories (Kisslegg, Germany) or were retired breeders from our own animal facility. Mice with a cardiomyocyte-restricted deletion of STAT3 (STAT3 KO) were generated by breeding STAT3-floxed mice (STAT3(flox)) with α-myosin heavy chain promoter/Cre recombinase transgenic mice (αMHC-Cre<sup>Wt</sup>).11

In Vivo Mouse Model

Female C57Bl6/J mice of different ages (young, <3 months; aged, >13 months) and young female STAT3 KO mice were subjected to 30 minutes of ischemia and 2 hours of reperfusion without (young, n=12; aged, n=10; STAT3 KO, n=8) or with iPoco by 3 cycles of 10 seconds of ischemia and 10 seconds of reperfusion (iPoco×10:...
The area at risk was not different between groups (young ischemia/reperfusion, 37.6±2.6; young iPoco3×10, 34.4±1.8; young iPoco5×5, 36.7±2.4; aged ischemia/reperfusion, 36.8±2.8; aged iPoco3×10, 37.5±3.9; aged iPoco5×5, 38.0±3.0; STAT3 KO ischemia/reperfusion, 35.0±2.8; STAT3 KO iPoco3×10, 27.4±5.2; STAT3-KO iPoco5×5, 29.3±2, young ischemia/reperfusion+AG-490, 36.8±4.7; young iPoco3×10+AG-490, 29.9±6.7). The IS (in percentage of the area at risk) was reduced in young C57Bl6/J mice from 67.3±3.1 in controls to 50.2±2.9 by iPoco3×10 and to 49.8±3.8 by iPoco5×5 (Figure 1). In aged C57Bl6/J mice, however, only iPoco5×5 was effective in reducing the IS (46.5±4.2), and there was no difference in the IS between control mice (64.1±3.2) and mice undergoing iPoco3×10 (66.4±3.0). Young mice with a cardiomyocyte-restricted deletion of STAT3 (STAT3 KO) underwent ischemia/reperfusion alone or iPoco (iPoco3×10 and iPoco5×5). The IS was significantly reduced by iPoco5×5 (55.0±2.8), but not by iPoco3×10 (62.2±3.5) compared with control hearts (64.2±3.1, Figure 1).

The impact of STAT3 phosphorylation on cardioprotection by iPoco was analyzed by subjecting young AG-490–treated mice to ischemia/reperfusion alone or to iPoco×10. The IS was slightly, but not significantly reduced by AG-490 alone.
(56.9±5.7, n=7; Figure 2), but iPoco3×10 did not further reduce IS (52.6±5, n=6).

Western blot analysis on right ventricular proteins from C57Bl6/J mice showed a decrease of P-STAT3 in aged to 72.0±7.6% (P<0.05) and of total STAT3 to 72.1±5.5% (P<0.05) of that in young mice, which were set as 100% (Figure 3). The protein level of Cx43 in aged hearts was reduced to 70.3±7.4%.

To assess STAT3 phosphorylation induced by iPoco, young, aged, and AG-490–treated mice underwent 30 minutes of ischemia and 10 minutes of reperfusion with iPoco3×10. iPoco3×10 induced an increase in the ratio of P-STAT3/STAT3 in the postconditioned AWs of young vehicle-treated mice (1.62±0.27 arbitrary units) compared with the control PWs (0.79±0.18 arbitrary units; P<0.05) of the left ventricle (Figure 4). Whereas a trend toward an increase of P-STAT3/STAT3 in the AWs compared with the PWs was found both in aged and AG-treated C57BL6/J mice, the ratio of P-STAT3/STAT3 in the AWs (aged, 0.89±0.17 arbitrary units; young AG-490–treated, 0.96±0.28 arbitrary units) was significantly reduced compared with the AWs of young vehicle-treated mice.

**Discussion**

The present study demonstrates that the postconditioning algorithms iPoco3×10 and iPoco5×5 are equally effective in reducing IS in young C57Bl6/J mice. In aged C57Bl6/J mice and STAT3 KO mice, only iPoco5×5 was cardioprotective. The inhibition of JAK-2 by AG-490 abolished the IS reduction by iPoco3×10. Furthermore, iPoco3×10 induced a
phosphorylation of STAT3, whereas in the aged heart and in the AG-490–treated heart, the amount of P-STAT3 protein was reduced.

iPoco has been first described in a dog model, however, it is also effective in mice, rats, rabbits, pigs, and humans (reviewed elsewhere13). For cardioprotection by iPoco, both the number of postconditioning cycles and, perhaps more importantly, the duration of reocclusion appear to be important (for review see elsewhere14). In isolated buffer-perfused mouse hearts, 6 postconditioning cycles (10 seconds of ischemia and 10 seconds of reperfusion) were more effective in improving postischemic systolic and diastolic function than 3 postconditioning cycles.15 The occurrence of protection was proposed to depend on a delay in adenosine washout. However, in in situ rat hearts, IS reduction with iPoco by 3 cycles of 10 seconds of ischemia and 10 seconds of reperfusion was similar.16 When analyzing the impact of the duration of the postconditioning stimuli in rat hearts in situ, 6 cycles of 30 seconds of ischemia and 30 seconds of reperfusion did not reduce IS, whereas shortening of the postconditioning cycles to 10 seconds of ischemia and 10 seconds of reperfusion induced a significant IS reduction compared with ischemia/reperfusion alone.17 The benefit of more and shorter postconditioning cycles has been confirmed in isolated rabbit hearts, in which postconditioning with 6 cycles of 10 seconds of ischemia and 10 seconds of reperfusion was more effective in reducing IS than 4 cycles of 30 seconds of ischemia and 30 seconds of reperfusion.18 In the present study, we compared the protective effects of iPoco3/H11003 and iPoco5/H11003 and found a similar IS reduction in young C57Bl6/J mice. However, in aged C57Bl6/J mice, only iPoco5×5 was effective in reducing IS. This indicates that with increasing age, the intrinsic capacity of the heart to respond to a postconditioning stimulus is impaired and a different postconditioning stimulus is needed to achieve a significant IS reduction. It has already been demonstrated that iPoco does not confer cardioprotection under all pathological situations, because iPoco failed to reduce IS in hypercholesterolemic rabbits.19 The results of the present study add to the above findings in that varying the postconditioning algorithm does not modify IS reduction in hearts of young mice but that the protection obtained in aged or genetically modified hearts depends on the postconditioning protocol. Therefore, one can speculate that a certain redundancy in signaling pathways leading to cardioprotection exists that can be recruited by
different postconditioning algorithms but that such redundancy is of minor importance in young (primarily healthy) hearts but becomes more important in aged or diseased (genetically modified) hearts.

The importance of STAT3 for the signal transduction cascade of IP is well established.\(^8\)-\(^10\) In the present study, we addressed the question of whether or not STAT3 also has an impact on cardioprotection by iPoco. Indeed, iPoco\(_{3\times10}\) induced an increase in STAT3 phosphorylation. This is similar to IP, in which an enhancement of STAT3 phosphorylation has been described.\(^8\)\(^9\) The inhibition of JAK-2 by AG-490 caused a decrease in the STAT3 phosphorylation in the postconditioned AW of the left ventricle.

When studying the impact of AG-490 treatment on IS in young C57Bl6/J mice, we found a slight but not significant IS reduction by AG-490 in mouse hearts undergoing ischemia/reperfusion alone. An even more pronounced reduction in IS by AG-490 was described in rat hearts subjected to ischemia/reperfusion,\(^20\) whereas in mice infarct development following 30 minutes of ischemia and 24 hours of reperfusion remained unaffected by AG-490 treatment.\(^8\) Because AG-490 inhibits JAK-2 and thereby not only STAT3 but also other downstream protein kinases (for example, mitogen-activated protein kinases), this may help to explain its varying effect on infarct development per se. However, and most importantly, iPoco\(_{3\times10}\) failed to further reduce IS in AG-490-treated hearts, demonstrating the importance of STAT3 phosphorylation for cardioprotection by iPoco.

To directly study the impact of STAT3 for cardioprotection by iPoco, STAT3-deficient mice were subjected to ischemia/reperfusion alone or iPoco\(_{3\times10}\) and iPoco\(_{5\times5}\). In agreement with the pharmacological data, cardioprotection by iPoco\(_{3\times10}\) also was abolished in STAT3-deficient hearts. Therefore, the present study shows, for the first time, that STAT3 is not only involved in the cardioprotection by IP but also in the IS reduction by iPoco.

Because cardioprotection by iPoco\(_{3\times10}\) was lost in the aged mouse heart, we analyzed the STAT3 protein level in myocardium of mice older than 13 months. The first evidence for a decrease of STAT3 in the process of aging comes from the aged rat brain.\(^21\) In the present study, a decrease of phosphorylated and total STAT3 was detected in right ventricular proteins extracts derived from aged compared with young hearts. The Cx43 protein level, which served as a control protein, was also reduced in C57Bl6/J hearts older than 13 months, as described previously.\(^1\) The lack of IS reduction by iPoco\(_{3\times10}\) in the aged mice cannot be attributed to the diminished protein level of Cx43 because in young heterozygous Cx43-deficient mice with a 50% reduction of the protein, iPoco\(_{3\times10}\) still reduced IS.\(^12\)

In the aged mouse heart, in which iPoco\(_{3\times10}\) is ineffective, the ratio of P-STAT3/STAT3 in the postconditioned AW of the left ventricle was reduced compared with young hearts. The reduced amount of P-STAT3 may impair the STAT3-mediated signaling cascade in the aged mouse myocardium that induces cardioprotection by iPoco.

Given the complexity of the signal transduction pathway(s), we cannot explain the differences between iPoco\(_{3\times10}\) and iPoco\(_{5\times5}\) in young versus aged versus STAT3 KO mice. Whereas we cannot prove causality between the reduction of P-STAT3 in the aged mouse heart and the loss of cardioprotection by iPoco, our data demonstrating increased STAT3 phosphorylation by iPoco\(_{3\times10}\) and loss of IS reduction in AG-490–treated mice and STAT3 KO mice strongly suggest an important role of STAT3 in the phenomenon of iPoco.

Taken together, whereas in young C57Bl6/J mice, IS reduction is not dependent on the mode of postconditioning, in old C57Bl6/J mice, only iPoco\(_{5\times5}\) induces cardioprotection. STAT3 is not only important for the cardioprotection by IP but also for iPoco. The reduced STAT3 protein level in the aged mouse heart may contribute to the loss of cardioprotection by iPoco.

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Disclosures

None.

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