A Female Way to Protect the Heart
Say NO to Calcium

Fabio Di Lisa

Both clinical and experimental observations support the concept that female hearts are less susceptible to myocardial injury caused by ischemia and reperfusion. A large body of evidence indicates that estrogen is involved in gender-related mechanisms of protection. The protective effect of 17-β-estradiol (E2) administration was described as a reduced extent of necrosis in rabbit hearts undergoing coronary occlusion followed by reperfusion. This initial observation, as well as the reduced myocardial injury in female hearts, has been confirmed in different animal species using various experimental protocols. Myocardial protection was shown to be associated with activation of estrogen receptors (ER), and specifically of ERα. Besides the well-established cytosolic/nuclear localization, or at least proteins recognized by anti-ER antibodies, have also been detected at the level of plasma membrane and mitochondria. However, the lack of a typical transmembrane domain in cytosolic ER casts doubts about these additional membrane receptors. The activated estrogen–receptor complex triggers the synthesis of specific mRNAs and the production of a number of proteins that are responsible for the various effects elicited in the different cell types. Along with these “genomic” effects, additional processes termed “nongenomic” or alternative occur rapidly and independently of protein synthesis.

Among the many pathways that can modify the susceptibility to ischemic injury in female hearts, the relevance of nitric oxide (NO) signaling was addressed by Sun et al in this issue of Circulation Research. Increased NO formation underlies myocardial protection in females. Previous studies from Murphy’s group investigated gender differences in hearts subjected to ischemia reperfusion (I/R) protocols under conditions having elevated levels of intracellular Ca\(^{2+}\) in common. Invariably, on reperfusion a reduced degree of injury was detected in hearts from female rodents as compared with male littermates. Highlighting a pivotal role of NO generation in the protective mechanism, it was also noted that eNOS content was higher in female hearts consistent with estrogen induction of NOS expression. The NOS inhibitor L-NAME abolished the gender differences. NOS-mediated protection was linked to intracellular Ca\(^{2+}\) homeostasis by showing that, on isoproterenol addition, SR calcium loading was lower in female hearts. On the other hand, increased NO availability has been shown to decrease the activity of L-type Ca\(^{2+}\) channels. Therefore, it was hypothesized that the higher content of eNOS in female hearts might reduce the intracellular rise in Ca\(^{2+}\) by decreasing its entry through L-type Ca\(^{2+}\) channels. This hypothesis appears to be convincingly demonstrated by the study of Sun et al.

Confirming and extending previous reports, female mouse hearts pretreated with isoproterenol before ischemia displayed decreased injury as compared with hearts from male mice. In addition, besides a higher content of eNOS associated with caveolin-3 at the plasma membrane, in isoproterenol-pretreated female hearts ischemia caused n-NOS translocation from SR to sarcolemma. This finding confirms a previous report documenting intracellular redistribution of nNOS in infarcted hearts of senescent rats. The role of NOS in myocardial protection was supported not only by pharmacological approaches, but also by the absence of male–female differences in mice lacking eNOS or nNOS, suggesting that both isoforms have to be present to limit I/R-induced injury. This concept was supported by the assessment of S-nitrosothiol content that, reflecting an increased NOS activity, was higher in female wild-type mice, yet gender differences were absent in eNOS- and nNOS-null mice. Notably, in isoproterenol-treated hearts protection was observed only when S-nitrosothiol content was above 15 pmol/mg protein, suggesting that the combined activities of both eNOS and nNOS is required to provide high rates of S-nitrosothiol formation.

NOS Activation Antagonizes Intracellular [Ca\(^{2+}\)] Rise

To validate the link between increased NO formation and decreased intracellular Ca\(^{2+}\) accumulation, it was imperative to demonstrate that one or more proteins involved in Ca\(^{2+}\) homeostasis were S-nitrosylated, and that such a covalent modification was causally related to functional changes. Indeed, this was the case with the L-type Ca\(^{2+}\) channel α1 subunit that was identified as the predominant S-nitrosylated protein in membrane fractions. The degree of α1 subunit nitrosylation was increased in isoproterenol-treated hearts and even more when this treatment was followed by ischemia and reperfusion. This increase was larger in female than in male hearts from wild-type mice, whereas gender differences

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(Circ Res. 2006;98:298-300.)

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Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/01.RES.0000208091.94643.6f

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were abrogated by NOS inhibitors and were not detected in mice lacking eNOS or nNOS. Finally, this novel biochemical evidence of S-nitrosylation was causally correlated with a decreased function of L-type Ca\(^{2+}\) channels by showing that after isoproterenol addition females (1) have less of an increase in systolic Ca\(^{2+}\) under conditions where SR function was blocked, and (2) a reduced increase in SR Ca\(^{2+}\). In addition, the isoproterenol-induced increase in Ca\(^{2+}\) current was smaller in female than in male hearts, and again this difference was abrogated by NOS blockade. Of note, the direct assessment of Ca\(^{2+}\) current allows ruling out that the differences in Ca\(^{2+}\) levels between female and male hearts might be caused by other factors, such as action potential duration or Na–Ca exchanger activity. Future studies should identify the residue(s) that are S-nitrosylated in α1 subunit to add relevant information to the current understanding of structure-function relationships in L-type Ca\(^{2+}\) channels.

These findings provide a direct demonstration that the activity of L-type Ca\(^{2+}\) channel is decreased by its S-nitrosylation, a concept that was previously supported only by indirect evidence. This lends convincing support to a protective mechanism characterizing female hearts whereby an initial increased entry of Ca\(^{2+}\) stimulates the activity of NOS isoforms localized in the plasma membrane, so that the increased NO production can be easily targeted to the L-type Ca\(^{2+}\) channels. The consequent decrease in Ca\(^{2+}\) influx is likely to result in a reduced intracellular Ca\(^{2+}\) overload during ischemia, eventually favoring the maintenance of tissue viability on reperfusion. Such a sequence of events might represent a crucial mechanism underlying the well-established protection afforded by NO that has not yet been conclusively elucidated in molecular terms.\(^{23}\)

**Additional Protective Mechanisms in Female Hearts**

The inhibition of Ca\(^{2+}\) influx attributable to S-nitrosylation of L-type Ca\(^{2+}\) channels, though relevant, represents one of the many ways through which estrogen protects ischemic hearts. It must be pointed out that in the absence of isoproterenol treatment I/R injury was not different in female and male mice. It is tempting to speculate that stimulation by circulating estrogen, that is lacking in the perfused heart model used by Sun et al, might trigger additional protective mechanisms. These include long-term effects, such as synthesis of antioxidant and prosurvival proteins,\(^{17,24–26}\) and short-term responses, such as activation of PI3K/AKT.\(^{27,28}\) In particular, this latter process appears to reduce the formation of reactive oxygen species decreasing the activity of the proapoptotic p38α MAPK concomitant with upregulation of the prosurvival isoform p38β.\(^{29}\) Therefore, the NO-dependent mechanism of reduced Ca\(^{2+}\) accumulation elucidated by Sun et al\(^{19}\) is likely to contribute to a protective network activated by estrogen stimulation.

**Acknowledgments**

This work was supported by Grants from CNR, FIRB, and MIUR.

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**Key Words:** nitric oxide | calcium | ischemia | gender | S-nitrosylation | calcium
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_Circ Res_. 2006;98:298-300
doi: 10.1161/01.RES.0000208091.94643.6f

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/98/3/298