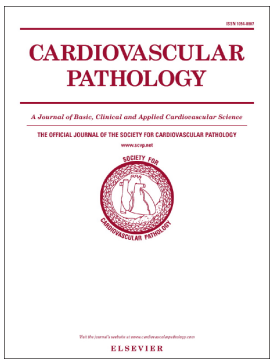

Survival Kinases-Dependent Pathways Contribute to Gender Difference in the Response to Myocardial Ischemia-Reperfusion and Ischemic Post-Conditioning

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PII: S1054-8807(17)30299-5
DOI: https://doi.org/10.1016/j.carpath.2017.12.003
Reference: CVP 7034

To appear in:

Received date: 7 November 2017
Revised date: 19 December 2017
Accepted date: 20 December 2017

Please cite this article as: Alejandro Ciocci Pardo, Sergio Scuri, Luisa F. González Arbeláez, Claudia Caldiz, Juliana Fantinelli, Susana M Mosca , Survival Kinases-Dependent Pathways Contribute to Gender Difference in the Response to Myocardial Ischemia-Reperfusion and Ischemic Post-Conditioning. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Cvp(2017), https://doi.org/10.1016/j.carpath.2017.12.003

SURVIVAL KINASES-DEPENDENT PATHWAYS CONTRIBUTE TO GENDER DIFFERENCE IN THE RESPONSE TO MYOCARDIAL ISCHEMIA-REPERFUSION AND ISCHEMIC POST-CONDITIONING

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Abstract

The response to ischemia/reperfusion and the effects of ischemic post-conditioning (IPC) are sex-dependent, but the mechanisms have not been clarified. Male (M) and female (F) rat hearts isolated and perfused using the Langendorff technique were subject to 30 min of global ischemia (GI) and 60 min reperfusion (R). In IPC hearts, three cycles of 30-sec GI/30-sec R were applied at the beginning of R. Infarct size and myocardial function were assessed. Superoxide production, antioxidant systems, and expressions of phosphorylated forms of serine/threonine kinase (Akt), glycogen synthase kinase 3β (GSK-3β), protein kinase C ε (PKCε), endothelial nitric oxide synthase (eNOS), and apoptosis were measured. In the basal state, superoxide production and apoptosis were lower, and antioxidant systems and phospho-kinase expressions were higher in F rather than in M hearts. After ischemia-reperfusion, infarct size was less in F hearts, and post-ischemic recovery of myocardial function was higher in F rather than in M hearts. Superoxide production, phospho-kinase activity, phospho-eNOS, and apoptosis increased in both sexes while antioxidants decreased in both sexes. After IPC, infarct size, superoxide production, and apoptosis decreased and phospho-eNOS increased in F and M hearts but phospho-kinase expressions and post-ischemic recovery of myocardial function improved only in M hearts. These results show that Akt/GSK-3β/PKCε/eNOS-dependent pathways-mediated superoxide production and apoptosis appear as important factors involved in the observed gender differences.

Key words: Male, female, ischemia-reperfusion, infarct size, ischemic post-conditioning

1. Introduction

Ischemic heart disease is the leading cause of death worldwide and accounts for almost half of the increased cardiovascular deaths. The influence of sex on the development of coronary artery disease has been widely examined [1]. Previous epidemiologic studies found similar or smaller infarct size and/or microvascular damage (no-reflow zone) after ischemia-reperfusion in women than in men [2-5]. At experimental level, most of observations performed on different animals, particularly the rat, confirm the presence of an increased resistance of the female myocardium to ischemia-reperfusion injury. Thus, a better recovery of post-ischemic contractile function and fewer arrhythmias were observed in female than age-matched males [6-10]. Differences in reactive oxygen species (ROS) generation by mitochondria [11, 12], in the expression of anti- and pro-apoptotic protein levels [13], and/or in the activation of cardioprotective kinases-dependent pathways were also detected [14-16]. Although these differences were previously attributed to sex hormones, the involved mechanisms remain unclear.

Ischemic post-conditioning (IPC), proposed by Zhao et al. [17], is a protection strategy in which multiple brief episodes of ischemia, applied at the beginning of reperfusion, improved heart outcomes. The protection exerted by IPC was demonstrated in different animal species and experimental protocols [18-20], including humans [21-23]. Very few studies tested the differences of IPC effectiveness between genders. They show contradictory results depending on the end-point considered (e.g., infarct size, contracture development, and post-ischemic systolic dysfunction, etc). Thus, Crisostomo et al. [24] reported the protective effect of IPC against post-ischemic dysfunction in isolated male rat hearts after either 20 or 25 min of ischemia, including female hearts only after 20 min of ischemia. Analyzing infarct size, Dow et al. [25] showed that IPC was ineffective in reducing it in female rat hearts. However, Penna et

al. [26] found a reduction of infarct size and post-ischemic dysfunction in female after IPC. Therefore, the results are inconclusive.

The purposes of this study were to analyze the response of isolated hearts from young adult male and female rats after 30 min of global ischemia and 60 min of reperfusion and to determine the effects of IPC in both sexes, examining the possible involved mechanisms.

2. Material and Methods

An expanded 'Material and Methods' section is available in Online Data Supplements.

2.1 Isolated rat heart preparation

Experiments were conducted in male and female 5-month-old Wistar rats. All procedures followed during this investigation were approved by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Medicine, University of La Plata following the Guide for the Care and Use of Laboratory Animals from the Institute for Laboratory Animal Research, National Research Council, Washington, D.C., National Academy Press, 2010 and/or European Union Directive for Animal Experiments 2010/63/UE.

Rats were anesthetized with ketamine-diazepam (80-5 mg/Kg). The heart was rapidly excised and perfused by the non-recirculating Langendorff technique.

2.2. Experimental protocols

After 20 min of stabilization, hearts from male (M) and female (F) were assigned to the following experimental protocols (Fig 1):

Non-ischemic control hearts (**NIC**; n = 5 for each sex): Hearts were perfused for 90 min.

Ischemic control hearts (**IC**; n = 7 for each sex): Hearts were subjected to 30 min of normothermic global ischemia followed by 1 hour of reperfusion. Global ischemia was induced by stopping the perfusate inflow line and the heart was placed in a saline bath held at 37°C.

Ischemic postconditioning (**IPC**, n = 7 for each sex): Three cycles of 30 sec of ischemia and 30 sec of reperfusion was applied at the beginning of reperfusion.

Additional hearts subjected to the same protocols (n = 6 for each one and for each sex) but stopping the perfusion at 53 min for NIC and at 3 min of reperfusion for IC and IPC were used for biochemical determinations.

2.3. Measurements

2.3.1. Infarct size

Infarct size was assessed by the widely validated triphenyltetrazolium chloride (TTC) staining technique and expressed as a percentage of area at risk [27].

2.3.2. Myocardial function

2.3.2.1. Systolic function

Myocardial contractility was assessed by the left ventricular developed pressure (LVDP) and maximal rate of rise of left ventricular pressure ($+dP/dt_{max}$).

2.3.2.2. Diastolic stiffness

Left ventricular wend diastolic pressure (LVEDP) was used as an index of ventricular diastolic stiffness.

2.3.2.3. Myocardial relaxation

To study the myocardial relaxation, the maximal rate of decay of left ventricular pressure ($-dP/dt_{max}$), the half time of relaxation (t50) and relaxation time constant (τ) were measured.

2.3.3. Coronary resistance

Coronary resistance was calculated as a quotient between coronary perfusion pressure and coronary flow and expressed as difference between the values obtained at the end of reperfusion period and that observed in the pre-ischemic period.

2.3.4. Biochemical determinations

2.3.4.1. Superoxide production

It was measured in cardiac strips using the lucigenin-enhanced chemiluminescence method and expressed as a.u./min/mg dry weight tissue [28].

2.3.4.2. Lipid peroxidation

The concentration of thiobarbituric reactive substances (TBARS) as an index of lipid peroxidation was measured by spectroscopic technique and expressed in nmol/mg protein [29].

2.3.4.3. Reduced glutathione content GSH)

It was determined by Ellman´s method and expressed as μg/mg protein [30].

2.3.4.4. Protein determination

The protein concentration was evaluated by the Bradford method [31].

2.3.4.5. *Immunoblotting*

A portion of M and F left ventricle was homogenized and resolved on SDS-PAGE, transferred to PVDF membranes and probed with antibodies anti-PSer9 GSK-3β, anti-PSer473-Akt, anti-PKCε, anti-Ser1177 eNOS, anti-nitrotyrosine, anti-MnSOD, anti-Bax, anti-Bcl2 and anti-caspase 3. GAPDH signal was used as a loading control.

2.4. Statistical analysis

Data are presented as mean ± SE and repeated measures of two-way analysis of variance (ANOVA) with Newman-Keuls test were used for multiple comparisons among groups. A P value < 0.05 was considered significant.

3. Results

3.1. Infarct size

Fig. 1 shows the infarct size in non-ischemic controls, ischemic controls, and post-conditioned hearts from M and F. In the absence of ischemia, and at the end of perfusion, infarct size was approximately 4 % of the risk area for both sexes. After 30 min of global ischemia and 1 h of reperfusion, the infarct size was significantly higher for M than F (40 ± 2% vs. 29 ± 4%). IPC significantly attenuated the infarct size in both sexes (16 ± 1% for M and 15 ± 1% for F).

3.2. Systolic and diastolic myocardial function

Non-ischemic control hearts exhibited a decrease in contractility of approximately 15-20 % at the end of perfusion. In ischemic-reperfused hearts, contractility decreased approximately 80 % with respect to pre-ischemic values in M and 45% in F. As shown in the Fig. 2, the recovery of systolic function was improved by IPC in M, but was not modified in F. At the end of the reperfusion period, LVDP (A panel), $+dP/dt_{max}$ (B panel) and $-dP/dt_{max}$ (C panel) reached higher values than those obtained in ischemic control hearts. In both sexes, the passive diastolic stiffness, assessed by LVEDP, increased in 30 min of global ischemia and increased more with reperfusion. At the end of reperfusion, LVEDP reached higher values in M than F hearts. IPC attenuated these increases only in M hearts.

In both sexes, t50 did not change during reperfusion, showing values of approximately 55 mseg. Only in M hearts, the relaxation time constant (τ) increased at 5 min of reperfusion and returned to pre-ischemic levels at its end (see Supplements).

3.3. Coronary resistance

Coronary resistance increased during reperfusion in M hearts. This increase (2.03 ± 0.32 mmHg/ml x min^{-1}) was significantly attenuated by IPC (0.90 ± 0.22 mmHg/ml x min^{-1}). In F hearts, coronary resistance did not show any change.

3.4. Superoxide production, TBARS concentration and nitrotyrosine expression

Superoxide production - assessed by lucigenin chemiluminescence - was higher in M than F in all experimental groups (Fig. 3A). In the basal state (a non-ischemic situation), superoxide production was lesser in F than M hearts. After ischemia-reperfusion, superoxide production increased in both sexes, which was reduced by IPC reaching non-ischemic levels only in M hearts. Lipid peroxidation - assessed by TBARS - was lower in F than M hearts in a non-ischemic situation (Fig. 3B). During ischemia-reperfusion, TBARS of F hearts increased and remained elevated after IPC. In contrast, M hearts exhibited high levels of TBARS in three situations (basal, ischemia-reperfusion, and IPC).

We evaluated protein nitration [produced by peroxynitrite (ONOO-), which resulted in a reaction between NO and superoxide ($O_2^{\cdot-}$)] through the measurement of 3-nitrotyrosine level by western blot. The expression of this protein increased in ischemic control hearts of both sexes and was significantly attenuated by IPC (Fig. 3C).

3.5. GSH and MnSOD expression

In the basal state, the content of GSH was markedly higher in F than M hearts. After ischemia-reperfusion, the level of GSH decreased only in F hearts and this value was not affected by IPC. In M hearts, the content of GSH did not change, showing a tendency to increase when IPC was applied (Fig. 4A). Under non-ischemic conditions, expression of the antioxidant enzyme MnSOD was higher in F than M hearts. After ischemia-reperfusion, though, the expression of MnSOD decreased in both sexes. In the

IPC group, the level of this enzyme increased in both sexes, reaching the highest values in M hearts (Fig. 4B).

3.6. P-Akt, P-GSK-3β, PKCε, and P-eNOS expression

Non-ischemic hearts from F showed a higher amount of the phosphorylated forms of Akt and GSK-3β than for M. At 3 min of reperfusion, following 30 min of global ischemia, the expression of P-Akt and P- GSK-3β increased approximately 25 % in both sexes, where higher values were found in F than M hearts. In post-conditioned hearts, kinase phosphorylation increased more in M (Fig. 5). The increases of P-eNOS in IC and IPC protocols were similar in both sexes.

3.7. Expression of Bax, Bcl2 and caspase 3

The level of pro-apoptotic Bax protein was significantly lower in F than M hearts in all experimental groups. However, after ischemia-reperfusion, Bax expression increased, and after IPC its level decreased in the hearts of both sexes. To the contrary, the expression of anti-apoptotic Bcl-2 was markedly higher in F than M hearts under non-ischemic, ischemic, and post-conditioning situations. Nevertheless, the Bcl-2 expression significantly decreased at 3 min of reperfusion in ischemic control hearts and increased in post-conditioned hearts from both sexes. The Bax/Bcl-2 ratio showed higher values in M than F hearts in all experimental groups. This ratio markedly increased early in the reperfusion after global ischemia and then decreased in post-conditioned hearts of both sexes. The level of caspase-3 - similar to Bax - showed higher values in M than F hearts, and increased during the first min of reperfusion and decreased in post-conditioned hearts (Fig. 6).

Discussion

In this study, we demonstrated that the application of a severe period of global ischemia (30 min) followed by 60 min of reperfusion to isolated hearts of young adult rats produced less damage in F compared to M. As such, we detected a smaller infarct size and diastolic stiffness, as well as an improvement in post-ischemic recovery of systolic function, isovolumic relaxation, and coronary perfusion in F compared to M hearts. We also found that ischemic post-conditioning decreased infarct size in both sexes, but attenuated myocardial dysfunction in M hearts.

Non-ischemic control

According to previous work, the contractility of F hearts in non-ischemic situations was similar [32] or lower than that demonstrated by M hearts [33-35]. In our experimental preparation, isolated hearts from F and M rats exhibited a similar basal contractility. However, we found differences in other analyzed parameters. For example, there was less superoxide production and lipid peroxidation, but a higher level of antioxidant systems (MnSOD and GSH) in F vs. M hearts. These results, in agreement with previous data [36, 37], indicate that in the basal state, F hearts have less oxidative damage than M hearts. This difference could be attributed to the previously reported sex differences in mitochondrial biogenesis, bioenergetics, and morphology [38]. F hearts also exhibited higher levels of phosphorylated forms of Akt, GSK-3β, and PKCε, as well as similar levels of phospho-eNOS and 3-nitrotyrosine than M hearts.

On the other hand, F hearts showed a higher level of anti-apoptotic Bcl-2 and a lower level of pro-apoptotic Bax, Bax/Bcl-2 ratio, and caspase-3 than M hearts. Therefore, in agreement with previous evidence [39], less myocyte apoptosis was found in F than M hearts.

These data suggest that the higher activation of Akt, GSK-3β, and PKCε-dependent signaling pathways could be contributing to lower superoxide production and pro-apoptotic factor release in F compared to M hearts.

Response to ischemia-reperfusion

Clinical studies demonstrated that myocardial infarct size may be similar [2] or smaller in women compared to men [4, 5]. However, most of the experimental observations confirmed that the F myocardium has increased resistance to ischemia-reperfusion injury [6-10]. In this study, F hearts showed a smaller infarct size and a better post-ischemic recovery of systolic and diastolic function compared to age-matched M hearts. The post-ischemic increase of coronary resistance was only observed in M hearts. We must ask which mechanisms are responsible for these differences. Oxidative stress and apoptosis play important roles in myocardial reperfusion injury. It has been described that early in reperfusion, there is an imbalance between ROS generation and antioxidant defenses, leading to oxidative stress [40-42]. In our experiments, during the first 3 min of reperfusion, the increase of superoxide production was greater in M than F hearts. Regarding antioxidant systems, MnSOD similarly decreased in both sexes, whereas GSH decreased only in F hearts. TBARS increased in F hearts, but this value was lower than that observed in M hearts. These data suggest F hearts suffered oxidative stress that was significantly lower than M hearts.

On the other hand, the oxidative stress is able to promote injury through a diminution of NO bioactivity [43]. In this study, hearts from both sexes showed a similar increase of 3-nitrotyrosine content, indicating that NO is not implicated in observed sex differences. Moreover, ROS directly and/or via redox-activated protein kinases (such as PKC) are able to modify the Ca^{2+} handling [44]. Both factors (ROS and Ca^{2+}) are implicated in the formation and/or opening of the mitochondrial permeability transition pore (mPTP),

which produces the release of pro-apoptotic factors and disturbance in mitochondrial function [45, 46]. In this sense, it was previously reported that F mitochondria are less prone to Ca^{2+} overload [47] and mPTP is less sensitive to Ca^{2+} [48]. In this study, the hearts from both sexes showed an increase of Bax and caspase-3 and a decrease of Bcl-2, with the changes more accentuated in F hearts. The expression of the phosphorylated forms of Akt, GSK-3β, and PKCε was higher in F than M hearts.

Therefore, the decrease of ROS production and pro-apoptotic factor release mediated by higher activation of Akt, GSK-3β, and PKCε-dependent signaling pathways could be responsible for the lower ischemia-reperfusion injury of F compared to M hearts.

Ischemic post-conditioning (IPC)

IPC has been widely demonstrated in different models, using hearts from M animals [18-20]. However, when IPC was applied to F hearts, the results are contradictory. Some studies report that IPC is able to reduce infarct size and post-ischemic systolic dysfunction, depending on ischemia duration [24, 26], whereas others demonstrate that IPC is ineffective [25, 49]. In our experiments, IPC decreased the infarct size in both sexes but improved post-ischemic recovery of myocardial function in M hearts. The increase of coronary resistance detected in M hearts was significantly attenuated by IPC, favoring the reestablishment of normal perfusion. As such, the question is: What are the possible explanations for these differences?

The benefits afforded by IPC involve the activation of multiple intermediate kinase pathways converging on mitochondria [50-52]. A reduction of ROS generation has been recognized as a possible IPC-mediated cardioprotective mechanism [53]. In our experiments, IPC produced a greater diminution of superoxide production and a higher increase in MnSOD expression in M than F hearts. The expression of P-Akt, P-GSK-3β, and P-PKCε increased in M, but did not change in F hearts. These data suggest that

kinases in ischemic control hearts from F are at their maximum concentration and that IPC cannot increase them further. This result could explain the observed lack of protection by IPC against the post-ischemic contractile dysfunction in F hearts.

The content of P-eNOS increased and 3-nitrotyrosine levels decreased in both sexes. Undoubtedly, a high expression of P-eNOS linked to an attenuation of superoxide production led to an increased NO bioavailability. It was previously reported that NO produces S-nitrosylation of L-type Ca^{2+} channel, thus reducing the Ca^{2+} overload and the ischemia-reperfusion injury [54]. Therefore, NO appears to play an important role in the decreased cell death/infarct size found in post-conditioned hearts from both sexes. Simultaneously, these hearts showed a decrease of Bax and caspase-3 expression and an increase of Bcl-2 content. In this way, post-conditioned hearts exhibited less apoptosis than non-post-conditioned hearts. These IPC-mediated beneficial actions can also contribute to the improvement of post-ischemic contractility in M hearts, but it was not enough to overcome post-ischemic cardiac dysfunction in F hearts. This could be interpreted that F myocardium is initially more protected against ischemic insult than M hearts.

These data suggest that a reduction of pro-apoptotic factor release and ROS production mediated by Akt/GSK-3β/PKCε/eNOS-dependent pathways targeting mitochondria could be involved in the decreased cell death detected in F and M post-conditioned hearts.

Conclusions

Our data confirm the existence of sex differences in response to ischemia-reperfusion and ischemic post-conditioning. According to our data, the observed differences between post-conditioned and non-post-conditioned M and F hearts appear to be associated with the degree of activation of Akt/GSK-3β/PKCε/eNOS-dependent

pathways and their impact on mPTP (Fig. 7). These findings support the development of sex-specific therapeutic strategies for the management of acute coronary syndrome.

Conflict of Interest Statement

None

Acknowledgements

This study was supported by the Grant M-169 from the National University of La Plata of Argentina to Dr. S. Mosca.

References

[1] Steg PG, Greenlaw N, Tardif JC et al. Women and men with stable coronary artery disease have similar clinical outcomes: insights from the international prospective CLARIFY registry. Eur Heart J 2012; 33: 2831-40.

[2] Tomey MI, Mehran R., Brener SJ, et al. Sex, adverse cardiac events, and infarct size in anterior myocardial infarction: an analysis of intracoronary abciximab and aspiration thrombectomy in patients with large anterior myocardial infarction (INFUSE-AMI).Am Heart J 2015; 169(1):86-93.

[3] De Luca G, Parodi G, Sciagrà R, et al. Relation gender to infarct size in patients with ST-segment elevation myocardial infarction undergoing primary angioplasty. Am J Cardiol 2013; 111(7):936-40.

[4] Langhans B, Ibrahim T, Hausleiter J, et al. Gender differences in contrast-enhanced magnetic resonance imaging after acute myocardial infarction. Int J Cardiovasc Imaging 2013; 29(3):643-50.

[5] Canali E, Masci P, Bogaert J, et al. Impact of gender differences on myocardial salvage and post-ischemic left ventricular remodelling after primary coronary angioplasty: new insights from cardiovascular magnetic resonance. Eur Heart J Cardiovasc Imaging 2012; 13(11):948-53.

[6] Deschamps AM, Murphy E, Sun J. Estrogen receptor activation and cardioprotection in ischemia reperfusion injury. Trends Cardiovasc Med 2010; 20:73-8.

[7] Kolar F, Ostadal B. Sex differences in cardiovascular function. Acta Physiol (Oxf) 2013; 207:584-7.

[8] Murphy E, Steenbergen C. Gender-based differences in mechanisms of protection in myocardial ischemia-reperfusion injury. Cardiovasc Res 2007; 75(3):478-86.

[9] Ross JL, Howlett SE. Age and ovariectomy abolish beneficial effects of female sex on rat ventricular myocytes exposed to simulated ischemia and reperfusion. PLoS One 2012; 7(6):e38425.

[10] Bell JR, Bernasochi GB, Varma U, et al. Delbridge Sex and sex hormones in cardiac stress-Mechanistic insights J Steroid Biochem Mol Biol 2013; 137:124-35.

[11] Pavon N, Martinez-Abundis E, Hernandez L, et al. Sexual hormones: effects on cardiac and mitochondrial activity after ischemia-reperfusion in adult rats. Gender difference. J Steroid Biochem Mol Biol 2012; 132:135-46.

[12] Lagranha CJ, Deschamps A, Aponte A, et al. Sex differences in the phosphorylation of mitochondrial proteins result in reduced production of reactive oxygen species and cardioprotection in females. Circ Res 2010; 106:1681-91.

[13] Chen C, Hu LX, Dong T, et al. Apoptosis and autophagy contribute to gender difference in cardiac ischemia-reperfusion induced injury in rats. Life Sci 2013; 93(7):265-70.

[14] Edwards AG, Rees ML, Gioscia RA, et al. PKC-permitted elevation of sarcolemmal KATP concentration may explain female-specific resistance to myocardial infarction. J Physiol 2009; 587(Pt 23):5723-37.

[15] Bae S, Zhang L. Gender differences in cardioprotection against ischemia/reperfusion injury in adult rat hearts: focus on Akt and protein kinase C signaling. J Pharmacol Exp Ther 2005; 315(3):1125-35.

[16] Huang C, Gu H, Zhang W, et al. Testosterone-down-regulated Akt pathway during cardiac ischemia/reperfusion: a mechanism involving BAD, Bcl-2 and FOXO3a. J Surg Res 2010; 164(1):e1-11.

[17] Zhao ZQ, Corvera JS, Halkos ME, et al. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. Am J Physiol Heart Circ Physiol 2003; 54: H579-88.

[18] Zhao ZQ. Postconditioning in reperfusion injury: a status report. Cardiovasc Drugs Ther 2010; 24: 265-79.

[19] Hausenloy DJ, Yellon DM. Ischaemic conditioning and reperfusion injury. Nat Rev Cardiol 2016; 13: 193-209.

[20] Skyschally A, Van Caster P, Iliodromitis EK, et al. Ischemic postconditioning: experimental models and protocol algorithms. Basic Res Cardiol 2009; 104: 469-83.

[21] Staat P, Rioufol G, Piot C, et al. Postconditioning the human heart. Circulation 2005; 112: 2143-8.

[22] Safaei N, Sheikhalizadeh MA, Badalzadeh R. Effect of ischemic postconditioning on myocardial protection in patients undergoing coronary artery bypass grafting surgery with cardiopulmonary bypass. J Cardiovasc Thorac Res 2016; 8: 65-71.

[23] Araszkiewicz A, Grygier M, Pyda M, et al. Postconditioning reduces enzymatic infarct size and improves microvascular reperfusion in patients with ST-segment elevation myocardial infarction. Cardiology 2014; 129: 250-7.

[24] Crisostomo PR, Wang M, Wairiuko GM, et al. Postconditioning in females depends on injury severity. J Surg Res 2006; 134(2):342-7.

[25] Dow J, Kloner RA. Postconditioning does not reduce myocardial infarct size in an in vivo regional ischemia rodent model. J Cardiovasc Pharmacol Ther 2007; 12:153-63.

[26] Penna C, Tullio F, Merlino A, et al. Postconditioning cardioprotection against infarct size and post-ischemic systolic dysfunction is influenced by gender. Basic Res Cardiol 2009; 104(4):390-402.

[27] Suzuki M, Sasaki N, Miki T, et al. Role of sarcolemmal K(ATP) channels in cardioprotection against ischemia/reperfusion injury in mice. J Clin Invest 2002; 109: 509-16.

[28] Caldiz CI, Garciarena CD, Dulce RA, et al. Mitochondrial reactive oxygen species activate the slow force response to stretch in feline myocardium. J Physiol 2007; 584: 895-905.

[29] Buege JA, Aust SD. Microsomal lipid peroxidation. Methods Enzymol 1974; 52: 302-9.

[30] Sedlak J, Lindsay RH. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem 1968; 25:192-205.

[31] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248-54.

[32] Escudero EM, Orlowski A, Díaz A, Pinilla OA, Ennis IL, Aiello EA. Gender differences in cardiac left ventricular mass and function: Clinical and experimental observations. Cardiol J 2014; 21(1):53-9.

[33] Chu SH, Sutherland K, Beck J, et al. Sex differences in expression of Ca^{2+}-handling proteins and β-adrenergic receptors in rat heart ventricle. Life Sci 2005; 76: 2735-49.

[34] Parks RJ, Howlett SE. Sex differences in mechanisms of cardiac excitation-contraction coupling. Pflugers Arch Eur J Physiol 2013; 465: 747-63.

[35] Curl CL, Wendt IR, Kotsanas G. Effects of gender on intracellular [Ca^{2+}] in rat cardiomyocytes. Pflugers Arch Eur J Physiol 2001; 441:709-16.

[36] Borrás C, Sastre J, Garcia-Sala D, et al. Mitochondria from females exhibit higher antioxidant gene expression and lower oxidative damage than males. Free Radic Biol Med 2003; 34: 546-52.

[37].- Colom B, Oliver J, Roca P, et al. Caloric restriction and gender modulate cardiac muscle mitochondrial H_2O_2 production and oxidative damage. Cardiovasc Res 2007; 74: 456-65.

[38] Khalifa AR, Abdel-Rahman EA, Mahmoud AM, et al. Sex-specific differences in mitochondria biogenesis, morphology, respiratory function, and ROS homeostasis in young mouse heart and brain. Physiol Rep 2017; 5(6): e13125.

[39] Liu H, Pedram A, Kim JK. Oestrogen prevents cardiomyocyte apoptosis by suppressing p38α-mediated activation of p53 and by down-regulating p53 inhibition on p38β. Cardiovasc Res 2011; 89(1):119-28.

[40] Hausenloy DJ, Yellon DM. Myocardial ischemia-reperfusion injury: A neglected therapeutic target. J Clin Invest 2013; 123(1): 92-100.

[41] Gustafsson Å. B., Gottlieb R. A. Heart mitochondria: gates of life and death. Cardiovasc Res 2008; 77(2):334-43.

[42] Patil N, Chavan V, Karnik ND. Antioxidant status in patients with acute myocardial infarction. Indian J Clin Biochem 2007; 22:45-51.

[43] Jones SP, Bolli R. The ubiquitous role of nitric oxide in cardioprotection. J Mol Cell Cardiol 2006; 40: 16-23.

[44] Sag CM, Wagner S, Maier LS. Role of oxidants on calcium and sodium movement in healthy and diseased cardiac myocytes. Free Radic Biol Med 2013; 63:338-49.

[45] Görlach A, Bertram K, Hudecova S, et al. Calcium and ROS: A mutual interplay. Redox Biol 2015; 6: 260-71.

[46] Wong R, Steenbergen C, Murphy E. Mitochondrial permeability transition pore and calcium handling. Methods Mol Biol 2012; 810: 235-42.

[47] Chweih H, Castilho RF, Figueira TR. Tissue and sex specificities in Ca2+ handling by isolated mitochondria in conditions avoiding the permeability transition. Exp Physiol 2015;100(9):1073-92.

[48] Milerová M, Drahota Z, Chytilová A, et al. Sex difference in the sensitivity of cardiac mitochondrial permeability transition pore to calcium load. Mol Cell Biochem 2016; 412(1-2):147-54.

[49] Lee DS, Steinbaugh GE, Quarrie R, et al. Ischemic postconditioning does not provide cardioprotection from long-term ischemic injury in isolated male or female rat hearts. J Surg Res. 2010; 164(2):175-81.

[50] VintenJohansen J, Zhao Z-Q, Zatta AJ, et al. Postconditioning. A new link in nature's armor against myocardial ischemia-reperfusion injury. Basic Res Cardiol 2005; 100:295-310.

[51] Yang Y-M, Proctor JB, Cui L, et al. Multiple, brief coronary occlusions during early reperfusion protect rabbit hearts by targeting cell signaling pathways. J Am Coll Cardiol 2004; 44:1103-10.

[52] Gomez L, Thibault H, Gharib A, et al. Inhibition of mitochondrial permeability transition improves functional recovery and reduces mortality following acute myocardial infarction in mice. Am J Physiol Heart Circ Physiol 2007; 293: H1654-61.

[53] Sun HY, Wang NP, Kerendi F, et al. Hypoxic postconditioning reduces cardiomyocyte loss by inhibiting ROS generation and intracellular Ca^{2+} overload. Am J Physiol Heart Circ Physiol 2005; 288(4):H1900-8.

[54] Sun J, Picht E, Ginsburg KS, et al. Hypercontractile female hearts exhibit increased s-nitrosylation of the l-type Ca^{2+} channel alpha1 subunit and reduced ischemia/reperfusion injury. Circ Res 2006; 98 (3): 403-11.

LEGENDS

Figure 1: Upper panel: experimental protocols. NIC: non-ischemic control; IC: ischemic control; IPC: ischemic post-conditioning; GI: global ischemia. Lower panel: Typical slices of heart from both sexes stained with TTC and mean values of infarct size, expressed as a percentage of risk area, in NIC, IC and IPC groups of hearts from male and female rats. Observe that infarct size of female was lesser than male. IPC reduced the infarct size in both sexes reaching similar values. * $p < 0.05$ IPC vs. IC; # $p < 0.05$ female vs. male.

Figure 1S: Scheme of the experimental design, indicating on the left ventricular pressure (LVP) and its first derivative (dP/dt) traces the measured parameters [developed pressure (LVDP), end diastolic pressure (LVEDP), maximal rate of rise of LVP ($+dP/dt_{max}$), maximal rate of decay of LVP ($-dP/dt_{max}$), half time of relaxation (t50) and relaxation time constant (τ)].

Figure 2: Time course of left ventricular developed pressure (LVDP, A panel), maximal rate of rise of left ventricular pressure ($+dP/dt_{max}$, B panel) and maximal rate of decay of LVP ($-dP/dt_{max}$, C panel), expressed as percentage of preischemic values, and left ventricular end diastolic pressure (LVEDP), expressed in mmHg, in NIC, IC and IPC hearts from male (M) and female (F) rats. Note that post-ischemic recovery of myocardial function in F was higher while diastolic stiffness was lesser in F than in M hearts. IPC improved the post-ischemic contractility only in M. * $p < 0.05$ IC F vs. IC M and IPC M vs IC M.

Figure 2S: Time course of t50 (half time of relaxation) and τ (relaxation time constant) expressed in msec during reperfusion in NIC, IC and IPC hearts from male (M) and female (F) rats. Both parameters increased early in reperfusion only in M hearts and gradually returned to pre-ischemic values. IPC annulled these changes. * $p < 0.05$

Figure 3: Superoxide ($O_2^{\cdot -}$) production (A panel), thiobarbituric acid reactive substances concentration (TBARS, B panel) and expression of 3-nitrotyrosine (C panel) in NIC, IC and IPC hearts from male (M) and female (F) rats. In NIC group, the $O_2^{\cdot -}$ production and TBARS of F hearts were lesser than M ones. After ischemia-reperfusion (IC group) $O_2^{\cdot -}$ production increased in both sexes and TBARS increased only in F hearts. The expression of 3-nitrotyrosine was similar in NIC hearts from M and F and decreased in IC groups. In both sexes IPC decreased $O_2^{\cdot -}$ production and 3-nitrotyrosine but not modified TBARS content. * $p < 0.05$ F vs. M; # $p < 0.05$ vs. NIC; ϕ $p < 0.05$ IPC vs. IC.

Figure 4: Reduced glutathione content (GSH, A panel) and MnSOD expression in NIC, IC and IPC hearts from male (M) and female (F) rats. In NIC group, these parameters were higher in F hearts than in M ones. After ischemia-reperfusion (IC group) the level of GSH only decreased in F hearts and the values were not modified by IPC. The MnSOD expression decreased in IC hearts and increased in post-conditioned hearts in both sexes. * $p < 0.05$ F vs. M; # $p < 0.05$ vs. NIC; ϕ $p < 0.05$ IPC vs. IC.

Figure 5: Representative immunoblots of phosphorylated forms and summary of densitometry data of phospho-Akt (P-Akt, A panel), phospho-GSK-3β (P-GSK-3β, B panel), phospho-PKCε (P-PKCε, C panel) and phospho-eNOS (P-eNOS, D panel), in cardiac homogenate of NIC, IC and IPC hearts from male (M) and female (F) rats. The expression of P-Akt, P-GSK-3β, P-PKCε were higher in NIC hearts from F than in M ones, this level increased after ischemia-reperfusion reaching the highest values in F

hearts. IPC only increased the expression of those kinases in M hearts. P-eNOS was similar in F and M, increased in IC groups and increased more in IPC groups without sex differences. * $p < 0.05$ F vs. M; # $p < 0.05$ IC vs. NIC; ϕ $p < 0.05$ IPC vs. IC.

Figure 6: Expression of Bcl2, Bax, Bax/Bcl2 ratio and caspase 3 in cardiac homogenate of NIC, IC and IPC hearts from male (M) and female (F) rats. In NIC groups Bcl-2 was higher and Bax, Bax/Bcl2 ratio and caspase 3 were lesser in F than in M hearts. After ischemia-reperfusion, Bcl-2 decreased and Bax, Bax/Bcl2 and caspase 3 increased in both sexes. IPC normalized these parameters returning to the non-ischemic levels. * $p < 0.05$ F vs. M; # $p < 0.05$ IC vs. NIC; ϕ $p < 0.05$ IPC vs. IC.

Figure 7: Scheme of the important steps involved in the sex differences to ischemia-reperfusion. These steps are represented by the activation of Akt, GSK-3β, PKCε and eNOS-dependent pathways and their impact on mPTP formation and/or opening. Thus, when the activation of kinases is maximal (as occurs in female and after IPC in male) the mPTP formation is minimal and the consequent release of ROS and pro-apoptotic factors decreases leading to a reduction of the cell death.

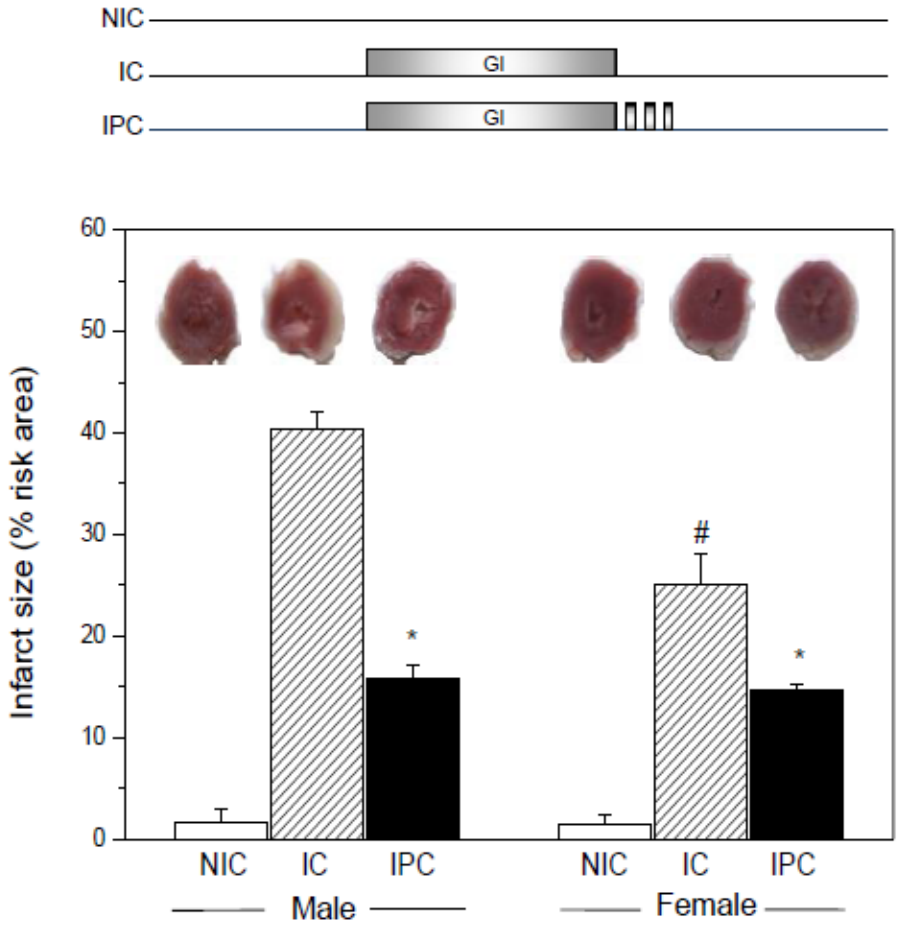


Figure 1

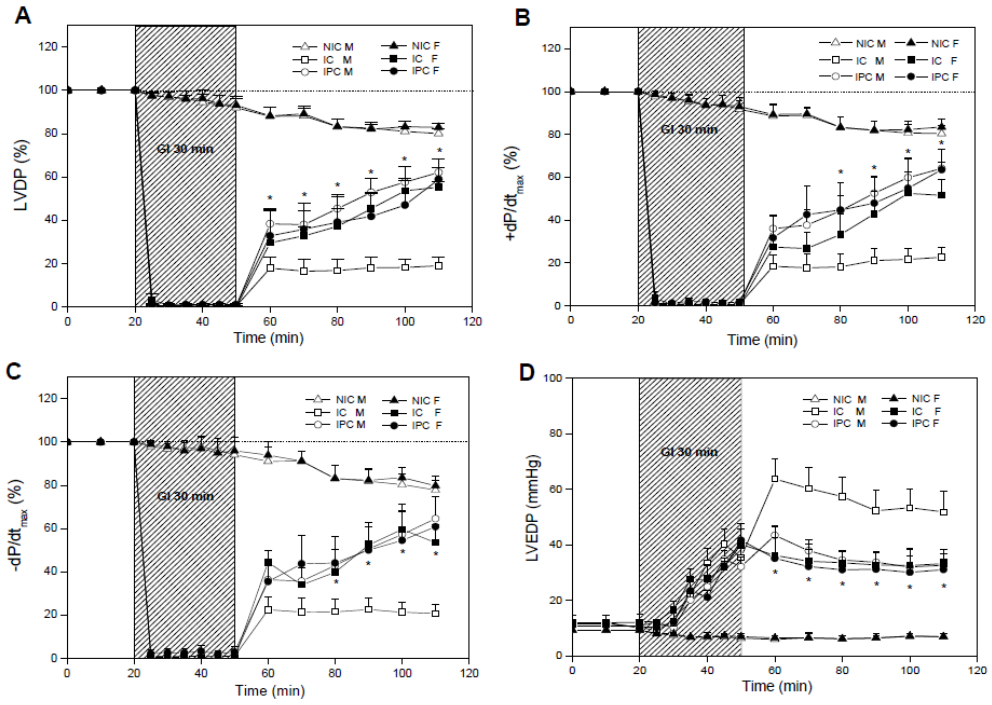


Figure 2

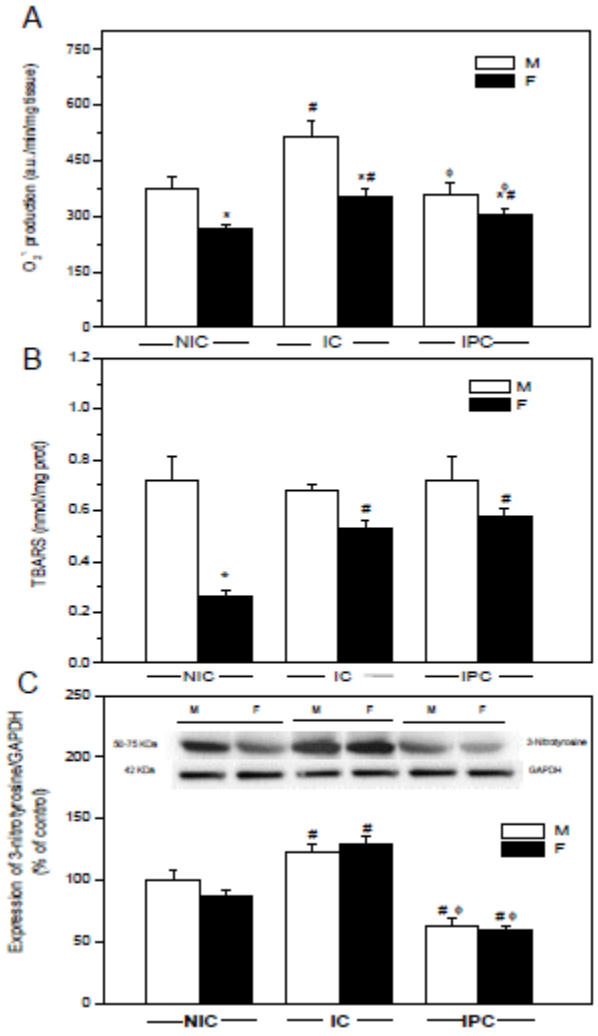


Figure 3

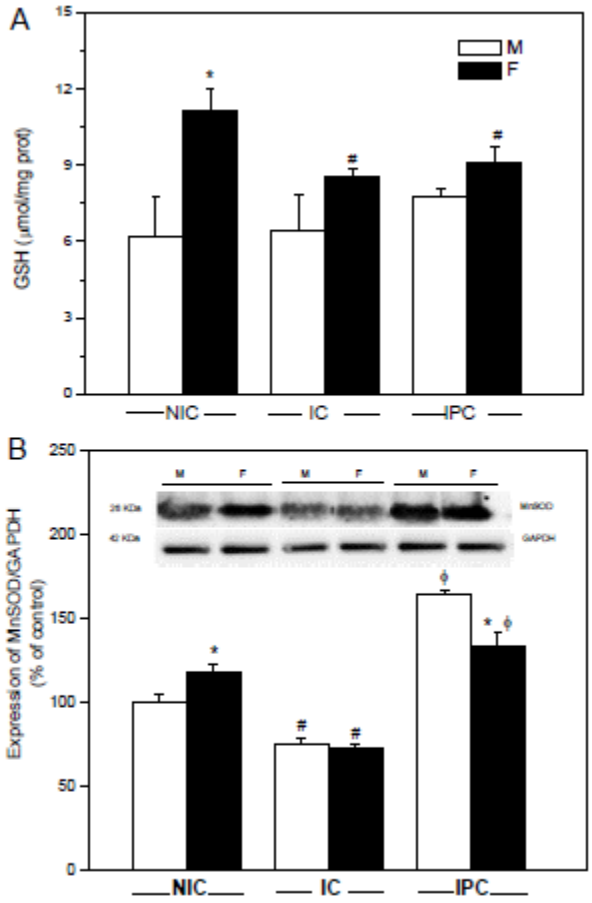


Figure 4

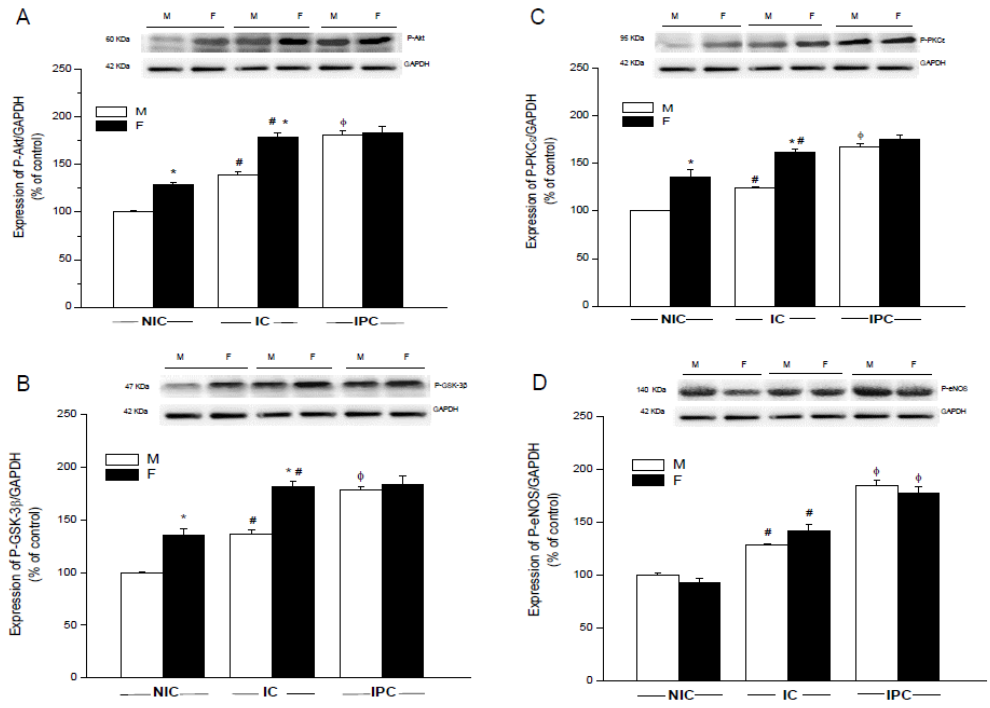


Figure 5

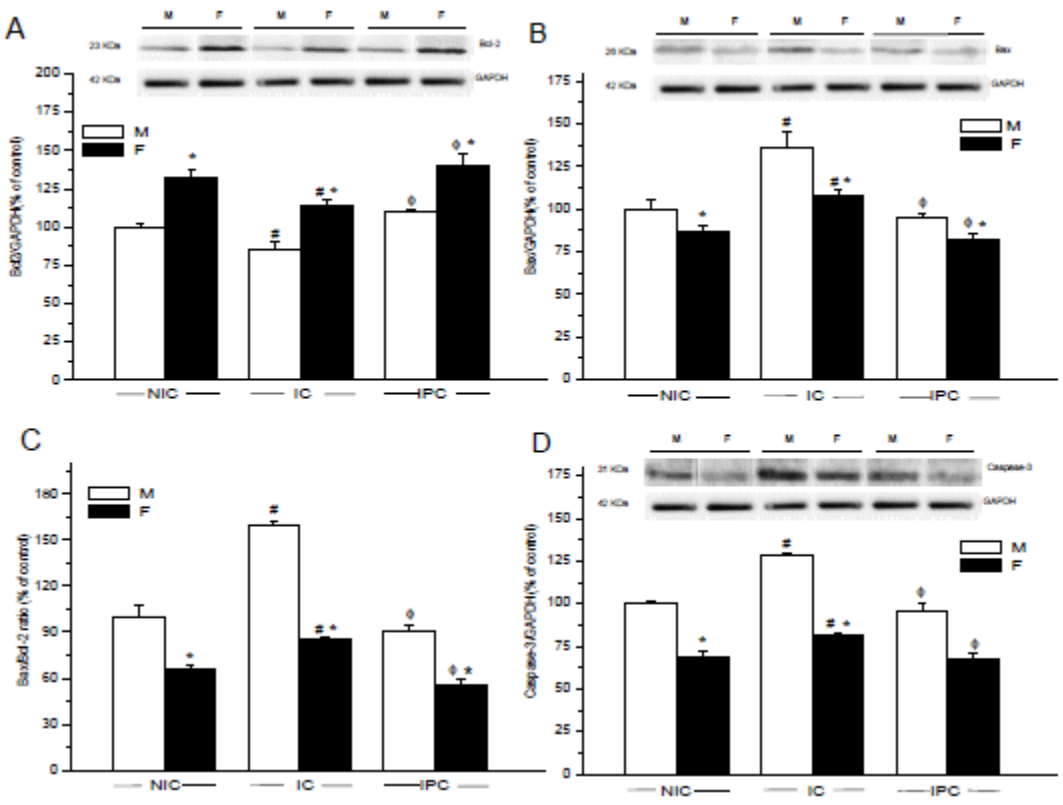


Figure 6

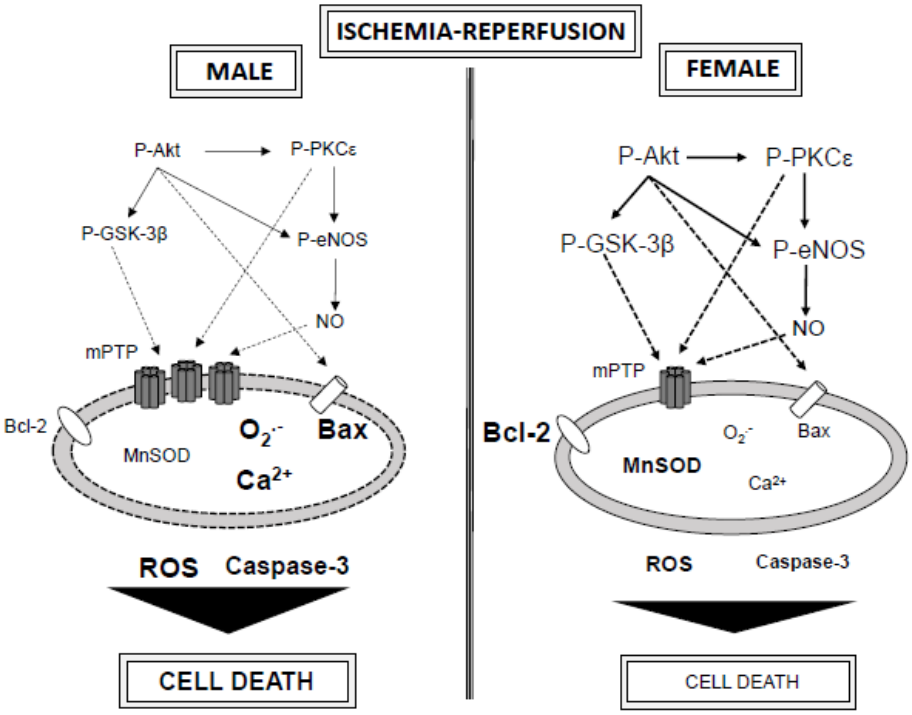
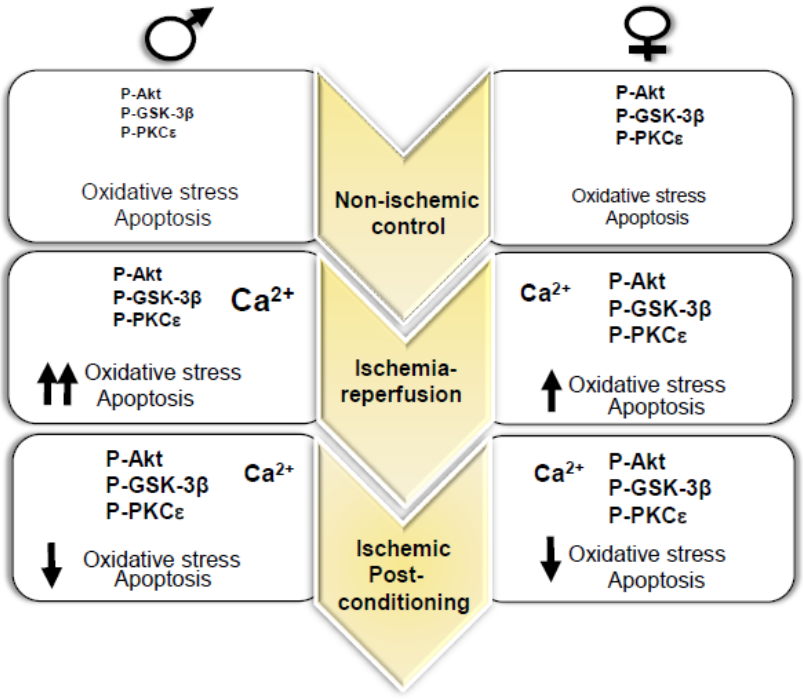


Figure 7



Graphical Abstract

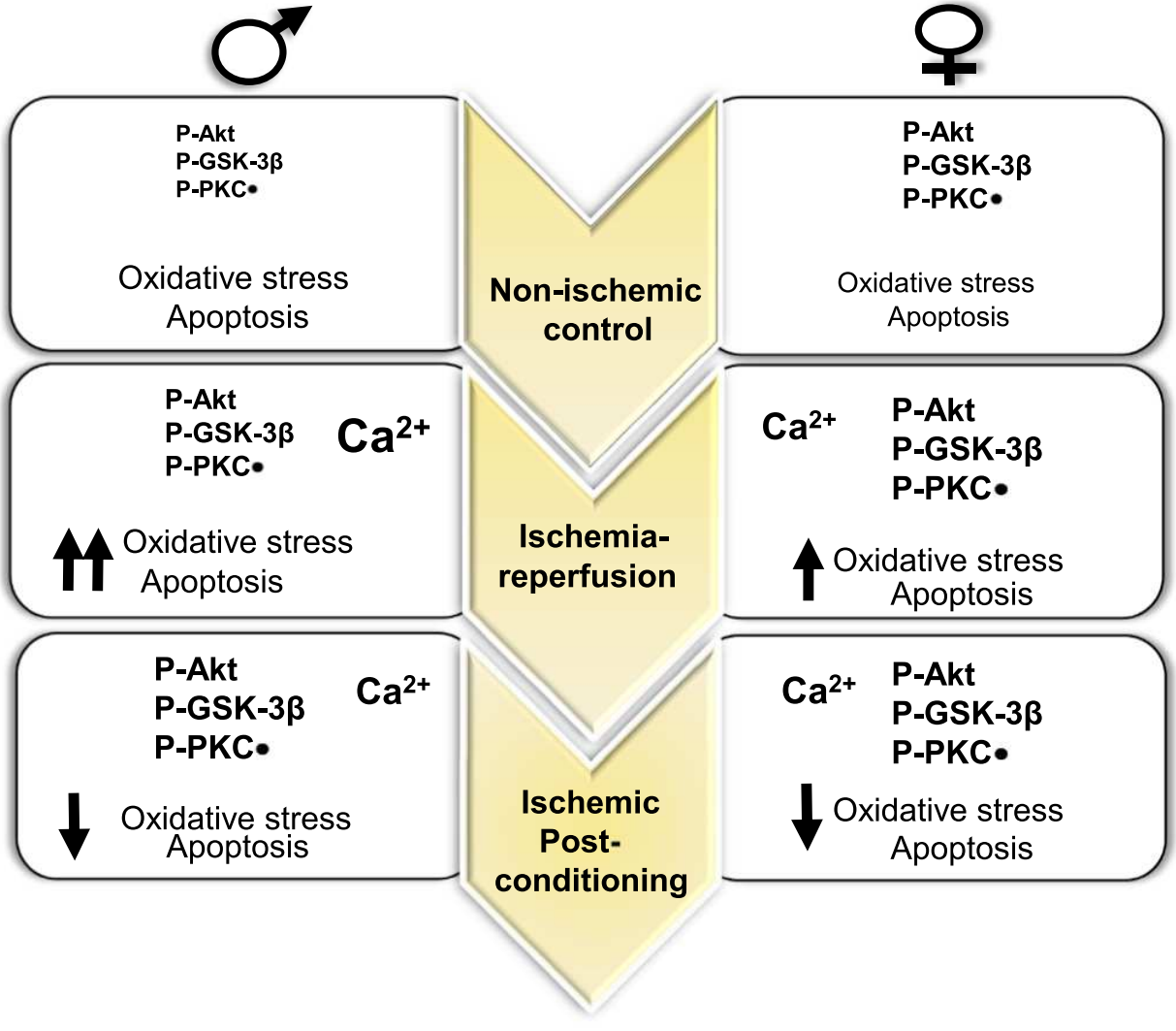
Highlights

1.- In non-ischemic situation female hearts exhibited higher levels of phosphorylated forms of Akt, GSK-3β and PKCε than male.

2.- After ischemia-reperfusion (IR) the infarct size, oxidative stress and apoptosis of female hearts were lesser than male.

3.- During IR female hearts showed the highest expression of P-Akt, P- GSK-3β and P-PKCε.

4.- The ischemic post-conditioning was more effective in male hearts.



Graphics Abstract

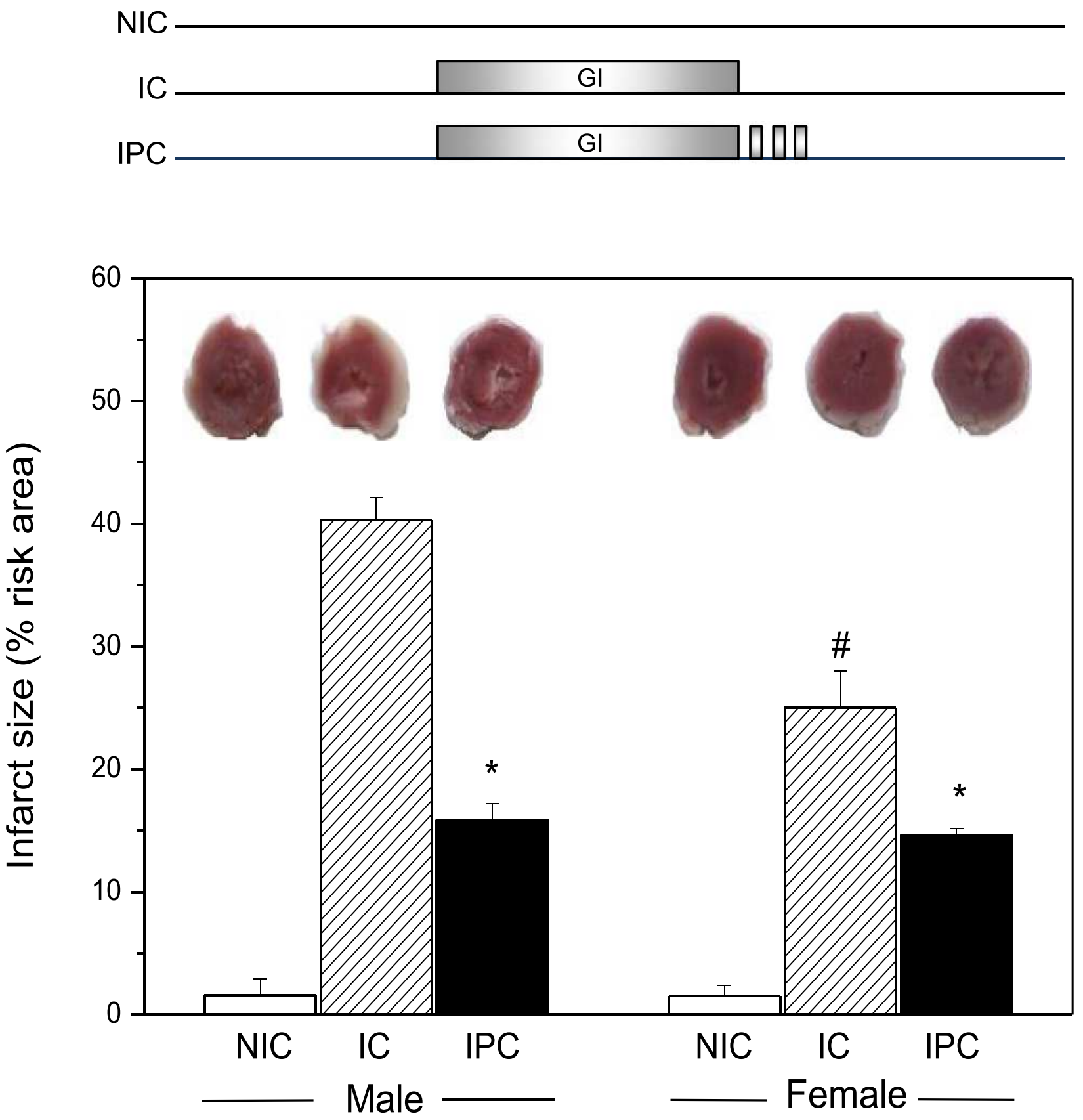

NIC
IC
GI
IPC
GI
60
50
40
30
20
10
0
Infarct size (% risk area)
*
#
*
NIC
IC
IPC
NIC
IC
IPC
Male
Female

Figure 1

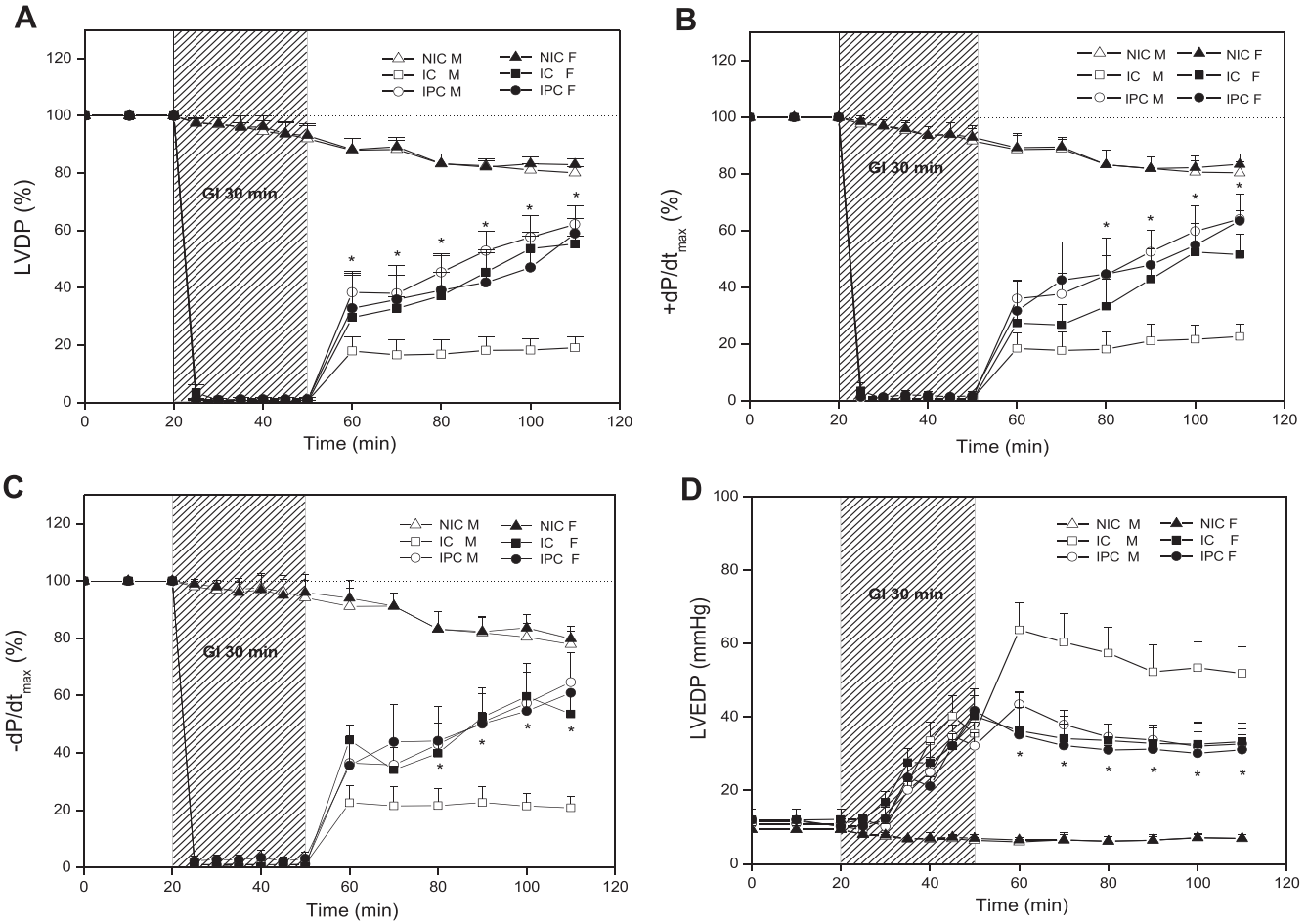

A
LVDP (%)
GI 30 min
NIC M
IC M
IPC M
NIC F
IC F
IPC F
Time (min)
B
+dP/dtmax (%)
GI 30 min
Time (min)
C
-dP/dtmax (%)
GI 30 min
Time (min)
D
LVEDP (mmHg)
GI 30 min
Time (min)

Figure 2

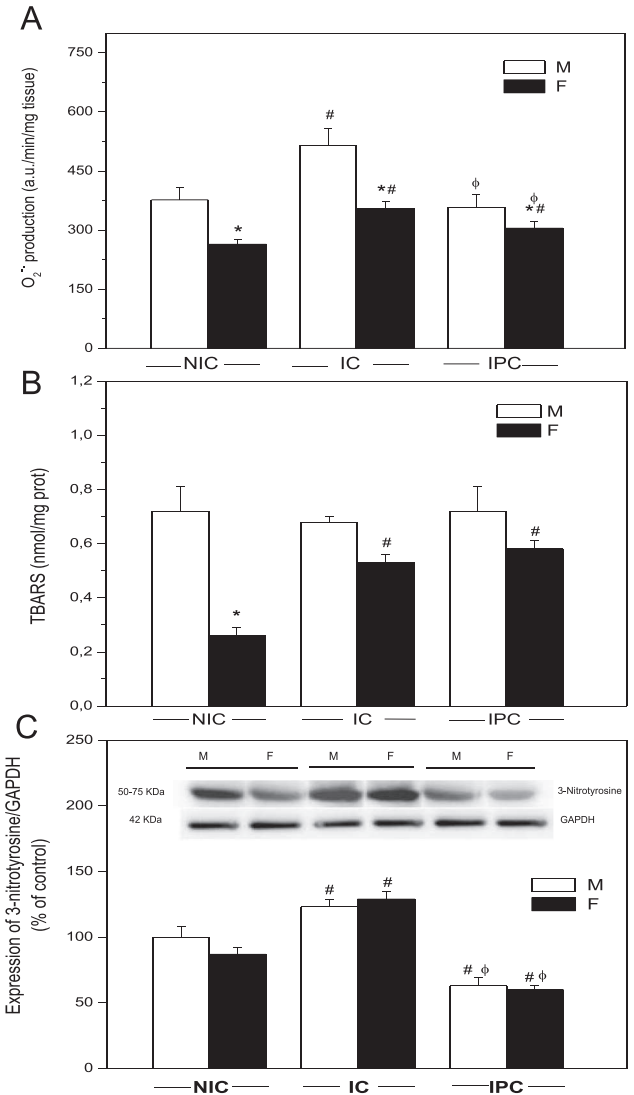

A
$O_2^{\cdot-}$ production (a.u./min/mg tissue)
750
600
450
300
150
0
M
F
#
*#
ϕ
ϕ
*#
*
NIC
IC
IPC
B
TBARS (nmol/mg prot)
1,2
1,0
0,8
0,6
0,4
0,2
0,0
M
F
*
#
#
NIC
IC
IPC
C
Expression of 3-nitrotyrosine/GAPDH (% of control)
250
200
150
100
50
0
M
F
M
F
M
F
50-75 KDa
42 KDa
3-Nitrotyrosine
GAPDH
M
F
#
#
ϕ
ϕ
NIC
IC
IPC

Figure 3

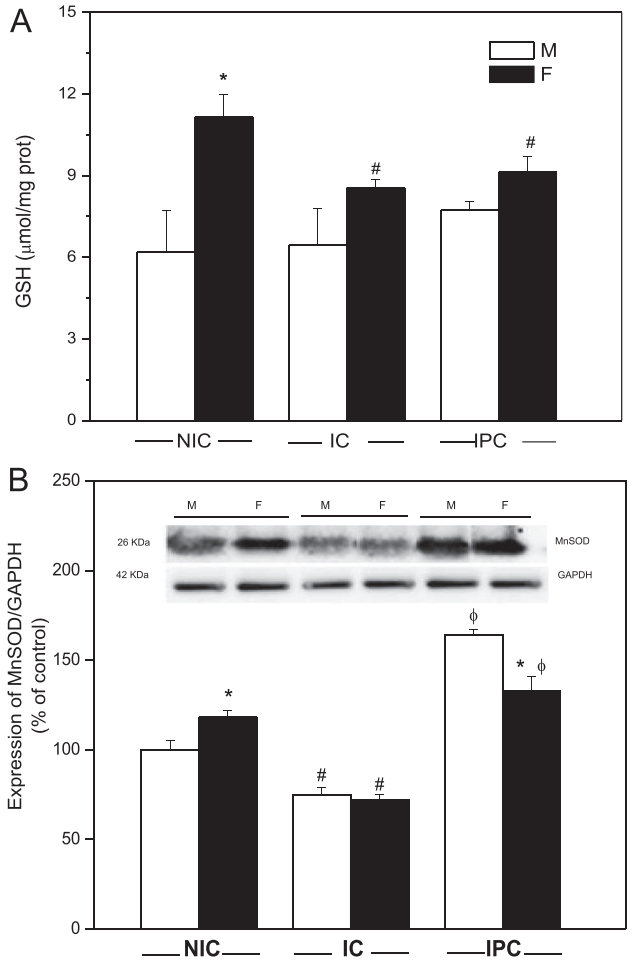

A
GSH (μmol/mg prot)
15
12
9
6
3
0
M
F
*
#
#
NIC
IC
IPC
B
Expression of MnSOD/GAPDH (% of control)
250
200
150
100
50
0
M
F
M
F
M
F
26 KDa
42 KDa
MnSOD
GAPDH
*
#
#
ϕ
* ϕ
NIC
IC
IPC

Figure 4

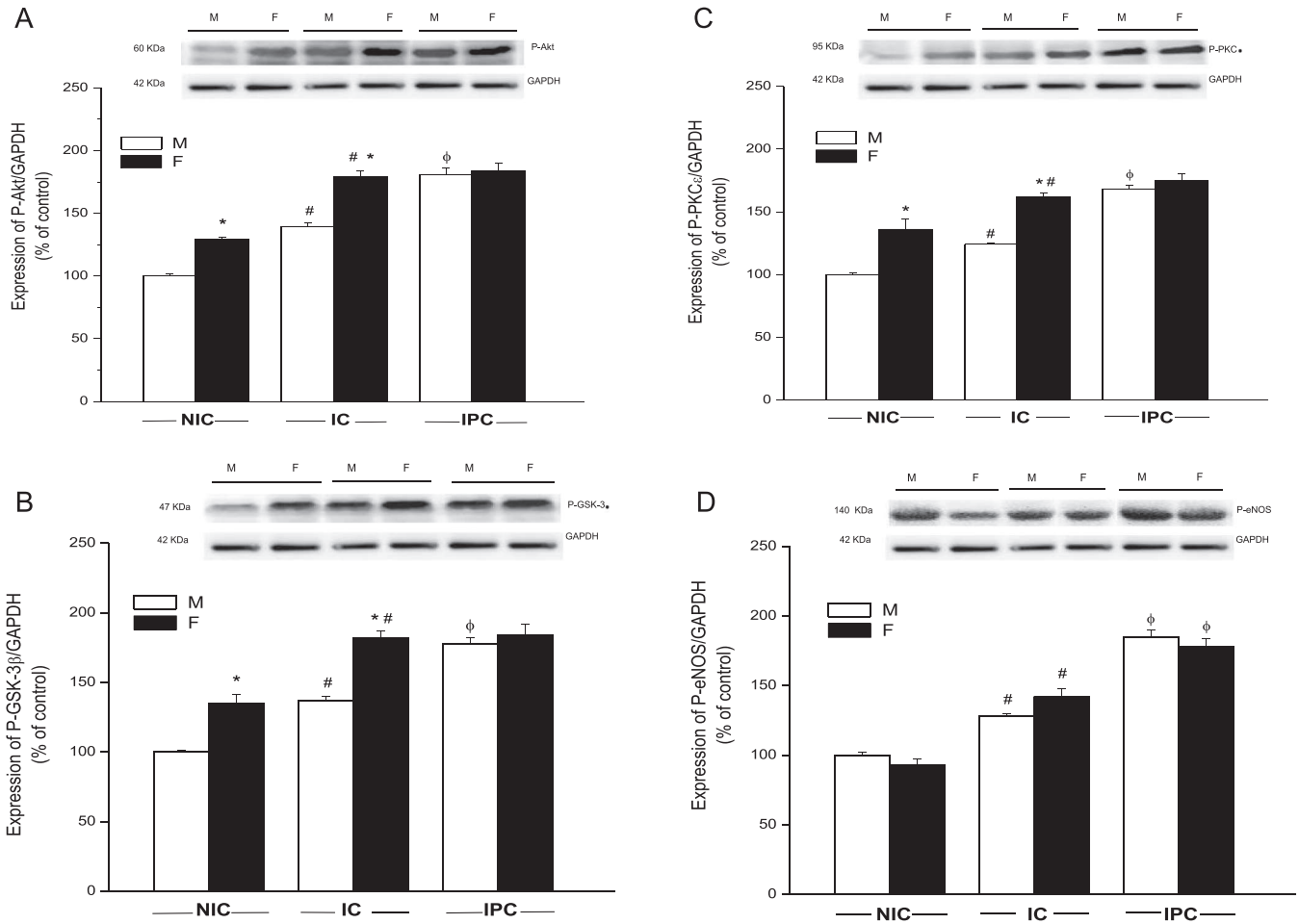

A
M F M F M F
60 KDa
P-Akt
42 KDa
GAPDH
Expression of P-Akt/GAPDH (% of control)
M
F
NIC
IC
IPC
B
47 KDa
P-GSK-3β
Expression of P-GSK-3β/GAPDH (% of control)
C
95 KDa
P-PKCδ
Expression of P-PKCδ/GAPDH (% of control)
D
140 KDa
P-eNOS
Expression of P-eNOS/GAPDH (% of control)

Figure 5

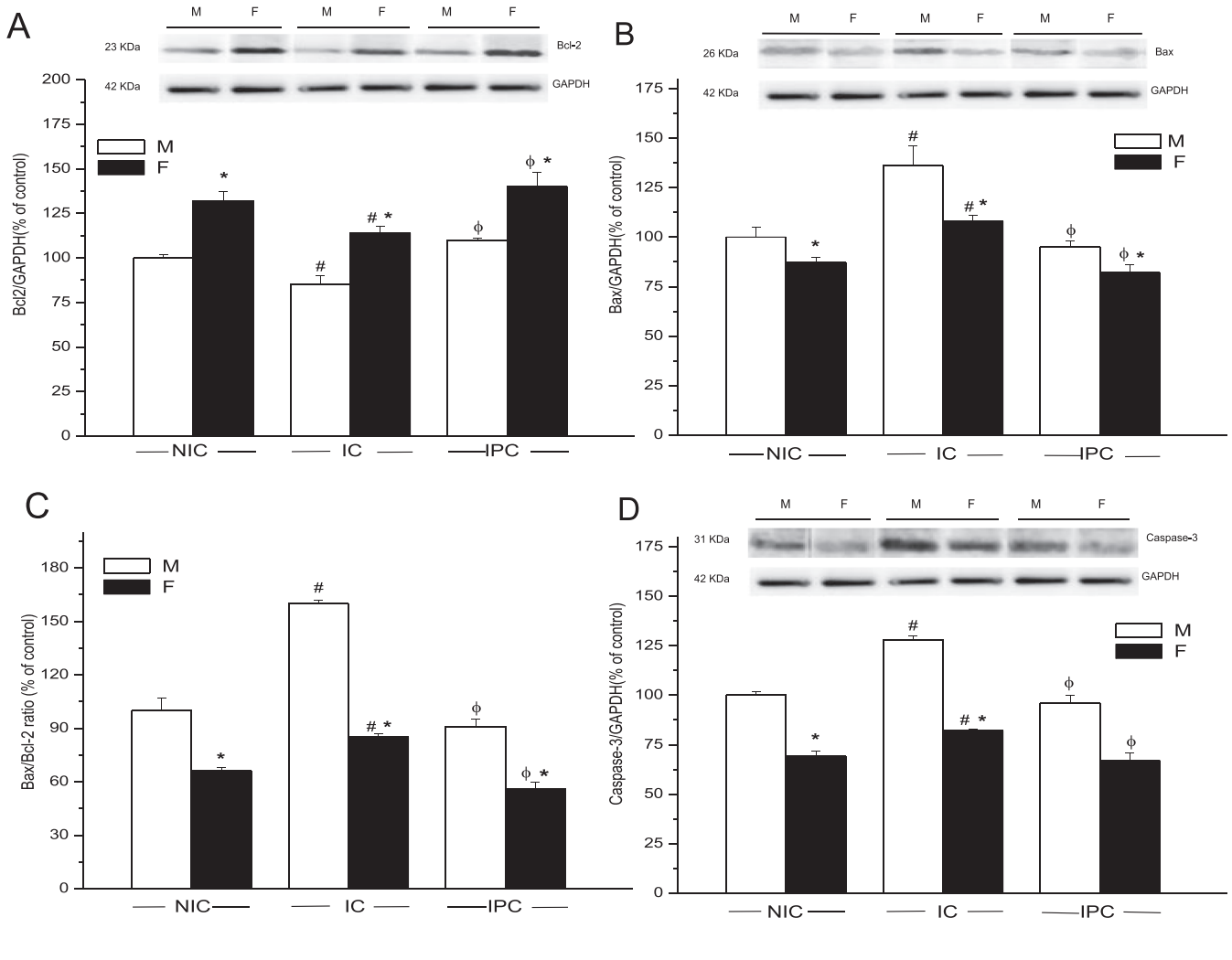

A
M F M F M F
23 KDa
Bcl-2
42 KDa
GAPDH
Bcl2/GAPDH(% of control)
M
F
NIC
IC
IPC
B
26 KDa
Bax
Bax/GAPDH(% of control)
C
Bax/Bcl-2 ratio (% of control)
D
31 KDa
Caspase-3
Caspase-3/GAPDH(% of control)

Figure 6

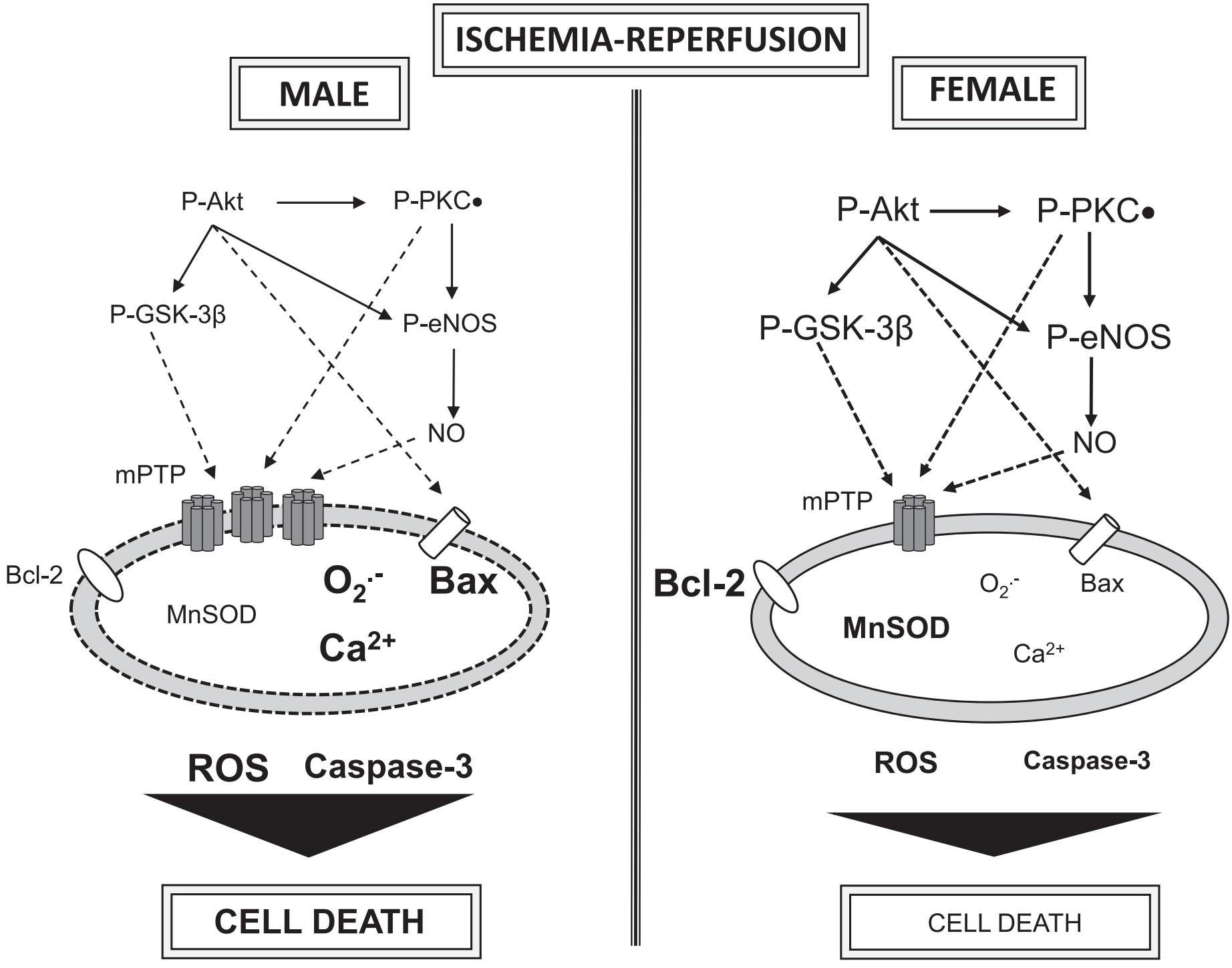

ISCHEMIA-REPERFUSION
MALE
FEMALE
P-Akt
P-PKC•
P-GSK-3β
P-eNOS
NO
mPTP
Bcl-2
MnSOD
$O_2^{\cdot-}$
Bax
Ca^{2+}
ROS
Caspase-3
CELL DEATH
P-Akt
P-PKC•
P-GSK-3β
P-eNOS
NO
mPTP
Bcl-2
$O_2^{\cdot-}$
Bax
MnSOD
Ca^{2+}
ROS
Caspase-3
CELL DEATH

Figure 7