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Energy status determines the distinct biochemical and physiological behavior of interfibrillar and sub-sarcolemmal mitochondria

Gino A. Kurian [a,*], Eduard Berenshtein [a], Or Kakhlon [b], Mordechai Chevion [a]

[a] Department of Cellular Biochemistry and Human Genetics, The Hebrew University-Hadassah Schools of Medicine and Dental Medicine, Ein Kerem, Jerusalem, Israel
[b] Department of Neurology, The Hadassah-Hebrew University Medical Center, Ein Kerem, Jerusalem, Israel

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ABSTRACT

Reports about the effect of ischemia and reperfusion on specific activities of the respiratory chain are often discrepant. One of the factors that govern this discrepancy is that typical mechanical procedures for mitochondrial isolation yield largely sub-sarcolemmal mitochondria (SSM), while the interfibrillar mitochondria (IFM), which provide most of the energy for the contractile apparatus, are under-represented. Here we investigated the impact of myocardial ischemia and reperfusion on SSM and IFM separately. Thirty-two Wister rats were randomly divided into four groups: control groups, ischemia groups, reperfusion groups and precondition groups. SSM and IFM were isolated from the rats' hearts from all the groups. The mitochondrial membrane potential ($\Delta\psi$) and swelling were assessed at energized (using either 5 mM succinate or 5 mM glutamate and 5 mM malate (GM) as a substrate) and non-energized conditions, where IFM showed better resistance to change in both conditions. Results showed that IFM have a higher coupling efficiency than SSM when energized by GM, but lower than SSM when energized with succinate. Preconditioning the rats' hearts prior to ischemia or reperfusion preserved the physiological and biochemical functions of both IFM and SSM and are energy dependent. The distinct physiological–biochemical functions of the mitochondrial sub-populations during ischemia and reperfusion depend on the overall energy status of the mitochondrial sub-population.

1. Introduction

Reperfusion injury is caused by the increased cellular Ca^{2+} and the reactive oxygen species (ROS) generation, both initiated in ischemia and amplified upon reperfusion. This injury originates mainly from the mitochondria [1]. Overall, it is thought that the combined effects of ROS generation and elevated Ca^{2+} play a critical role in the transition from reversible to irreversible reperfusion injury. In particular, they lead to the opening of the mitochondrial permeability transition pore (MPTP), now widely accepted to play a critical role in reperfusion injury [2]

Depending on their location in cardiomyocytes, heart mitochondria are classified as either sub-sarcolemmal (SSM) or interfibrillar (IFM) [3]. The two sub-populations are different with respect to both their inherited properties and their response to disease. An increased susceptibility of cardiac SSM to ischemic damage, as compared to IFM, has been observed in rat [4].

Lucas and Szweda [5] reported decreased respiration using NAD as a substrate after ischemia or reperfusion in the mitochondria isolated from the perfused Langendorff rats' hearts. On the other hand, other studies showed that there is no decrease in the respiratory activity in mitochondria after exposure to a 30 min ischemia [6], and NMR studies indicate that any putative post ischemic damage is not sufficient to slow down coupled electron flow [7]. Thus, it has been shown that after ischemia/reperfusion in heart, respiration may fall, rise or remain the same. We show that this uncertainty can be resolved by studying the effect of ischemia and reperfusion on distinct mitochondrial sub-populations, which can elucidate the contribution of mitochondrial dysfunction to cardiac failure.

2. Materials and methods

2.1. Animals and experimental design

Sprague–Dawley male rats (250–300 g) housed under standard conditions and fed regular *ad libitum* diet and water were used. All the experimental protocols were approved by the 'Institutional Animal Care and Use Committee' of the Hebrew University of Jerusalem, conforming to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85–23, revised 1996).

* Corresponding author. Address: School of Chemical & Biotechnology, SASTRA University, Thanjavur, India. Fax: +91 972 2 6415848.
E-mail addresses: ginokurian@hotmail.com, kurian@scbt.sastra.edu (G.A. Kurian).

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2.2. Perfusion protocols

Hearts were removed and mounted on the Langendorff apparatus as previously described by us [8].

2.3. Experimental groups

Rats were randomly divided into 4 main groups; control, ischemia (Isc.), reperfusion (I/R) and ischemic preconditioning (IPC) groups. The control group was divided into three subgroups, isolated hearts from these sub groups were subjected to continuous perfusion of Krebs Henseleit (KH) buffer for 25 min (P25), 60 min (P60) and 120 min (P120). Isolated rats' hearts were stabilized for 10 min in IPC and for about 25 min in the Isc. and the I/R to establish baseline parameters as a reference to the effects caused by subsequent manipulations. In the Isc. group, hearts were subjected to 35 min of global ischemia after stabilization. Similarly, in the I/R group, 35 min global ischemia was induced followed by 60 min of reperfusion. The ischemic preconditioning group was subdivided into three subgroups namely, IPC, IPC + I & IPC + IR. In the IPC group, after stabilization, the isolated rats' hearts were subjected to three cycles of 2 min ischemia followed by 3 min reperfusion. In the IPC +I and IPC +IR groups, the same procedure mentioned for IPC was followed but subsequently 35 min of global ischemia was induced in the IPC +I group whereas 35 min global ischemia followed by 60 min reperfusion was induced in IPC +IR groups.

2.4. Isolation of mitochondrial sub population

Rat heart mitochondria were isolated by differential centrifugation, essentially according to the method described by Palmer et al. [9]. IFM and SSM were purified using a 60% percoll gradient and Western blot analysis of HSP 60, the marker protein (data not included) against actin was used to determine the purity.

2.5. Mitochondrial oxidative phosphorylation

Oxygen consumption by mitochondria was measured using a Clarke type oxygen electrode at 37 °C. Mitochondrial respiration was measured with glutamate (10 mM) and malate (2 mM) as substrate or with succinate 10 mM in the presence of rotenone 0.5 μM.

2.6. Mitochondrial swelling

The mitochondrial swelling was determined by the rate of change in absorbance at 540 nm under energized (5 mM succinate or 5 mM glutamate plus malate (GM)) and non-energized conditions [10].

2.7. Mitochondrial membrane potential

Mitochondrial membrane potential was estimated as described [11] using the uptake of the positively charged fluorescent dye rhodamine 123.

2.8. Determination of infarct size

Infarct size (IS) was measured according to Mensah et al. [12] with minor changes. The percentage of infracted tissue (triphenyltetrazolium chloride negative) developed in the area at risk (AR) (Evans blue negative and TTC positive) was determined.

2.9. Glutathione assay

Total glutathione (GSH + GSSG) was measured according to the protocol described by Rahman et al. [13].

2.10. Statistical data analysis

The comparison between values of the same group, at various time points along the experiment was conducted using ANOVA. Differences in variables between the groups for a specific time point were analyzed using one-way ANOVA.

3. Results

3.1. Cardiac function and infarct size

The functional capacity of the heart during the experiment was assessed by the rate pressure product (RPP, product of the heart rate and left ventricular developed pressure). RPP of ischemia and I/R hearts were significantly lower and indicate poor recovery during the experimental procedure. The insult of ischemia reperfusion to IPC hearts significantly recovered the hearts as, compared to ischemia reperfusion controls (Table 1).

In order to show that the IPC procedure was effective as previously described [14] under our own conditions, infarct size was measured at the end of the protocols. As expected, infarct size was significantly decreased in preconditioned hearts as compared to their control (Table 2).

3.2. Mitochondrial respiration

In NADH-driven respiration through complex I, measured with GM as a substrate, the ADP/O ratio was decreased with perfusion time in both IFM and SSM (Fig. 1A). But when succinate was used as a substrate for respiration through complex II, the ADP/O ratio was increased with the perfusion time in IFM (Fig. 1B). Following ischemia and I/R, the ADP/O ratio in GM-driven respiration was reduced in both IFM and SSM as compared to the respective perfusion controls (Fig. 1A), however when succinate was used as substrate for respiration through complex II, only I/R groups showed reduction in both IFM and SSM. A lower rate of oxidative phosphorylation in SSM as compared to IFM in young rats has been reported elsewhere [15]. Similarly, in our study, the decrease in GM-driven oxidative phosphorylation (as estimated by the ADP/O ratio) prompted by both Isc. and I/R was more pronounced in SSM, where it was more than 20%, than in IFM (Fig. 1A). Tightness of coupling indicated by the respiratory control ratio (RCR) (RCR = state 3 respiration/state 4 respiration), was reduced in both sub-populations by reperfusion, with GM as respiration substrate (Fig 1C), indicating the possible damage induced by reperfusion as previously reported [16]. Mitochondrial sub-populations isolated from preconditioned rat's hearts improved the ADP stimulated respiration when compared to ischemia and ischemia–reperfusion controls (Fig 1A).

Table 1
Hemodynamics.

	EDP	DP	RPP
Perfusion 120 min ($n = 4$)	3 ± 2	97 ± 4	94 ± 2
I/R ($n = 7$)	46 ± 7	43 ± 4	33 ± 2
IPC +I/R ($n = 10$)	21 ± 5	89 ± 5	80 ± 2

Data represented as mean ± SE; EDP: end diastolic pressure; DP: developed pressure; RPP: rate pressure product. $^{*}p < 0.05$ vs control.

Table 2
Infarct size.

	Infarct size (% of total heart[a])
Perfusion 120 min (n = 4)	7 ± 0.7
I/R (n = 7)	21 ± 0.7
IPC +I/R (n = 10)	4 ± 0.9

Data are mean ± SE; $^{*}p < 0.05$ vs control.
[a] Since hearts were subjected to global ischemia, total cross sectional were defined as the total risk areas (Zhu 2007). Infarct size of each heart was the average of infracted area to total risk area ratio of all slices.

3.3. Mitochondrial swelling

We studied the swelling behavior of IFM and SSM in energized (GM or succinate) and non-energized conditions. Mitochondrial swelling was determined as the change in mitochondrial volume upon the supplementation of 200 μM calcium, which results in the decreased light scattering as the mitochondrial volume increases [17]. Cyclosporin, blocker of MPTP was used as a negative control to the calcium induced mitochondrial swelling, as previously reported [18]. IFM isolated from ischemic heart, but not SSM, showed swelling when they were energized with succinate, but relatively no swelling was observed in both sub-population with glutamate malate (GM) as substrate. Non-energized SSM from hearts subjected to ischemia exhibited significant swelling (Fig 2A). SSM from hearts exposed to ischemia–reperfusion showed higher swelling than IFM when energized with succinate (Fig 2C), while IFM displayed elevated swelling than SSM when GM was used as a respiration substrate (Fig 2B). Furthermore, non-energized SSM from heart subjected to I/R showed significant swelling as compared to their controls. CsA blocked the swelling in all cases (data not shown) indicating swelling was MPTP dependent. Ischemic preconditioning did not prevent the swelling of non-energized SSM (compared to P25group), but it restrained swelling in both IFM and SSM during ischemia and ischemia–reperfusion conditions (Fig 2A). Preconditioning the energized mitochondrial subpopulations prevented the swelling (Fig 2B).

3.4. Mitochondrial permeability transition

Mitochondrial swelling can cause membrane depolarization (proton leak will make the matrix more positive) as a result of MPTP opening [19]. MPTP opening can thus be detected by measuring the permeability of the inner mitochondrial membrane to the positively charged fluorescent dye rhodamine 123. In this study, non-energized IFM showed membrane hyper-polarization during ischemia and ischemia–reperfusion compared to P60 and P120 (Fig. 3A), while non-energized SSM exhibited depolarization (Fig. 3A) in all the experimental groups except for IPC. In energized mitochondria, SSM showed no significant change in all the experimental groups as compared to its' control (Fig. 3B and C).

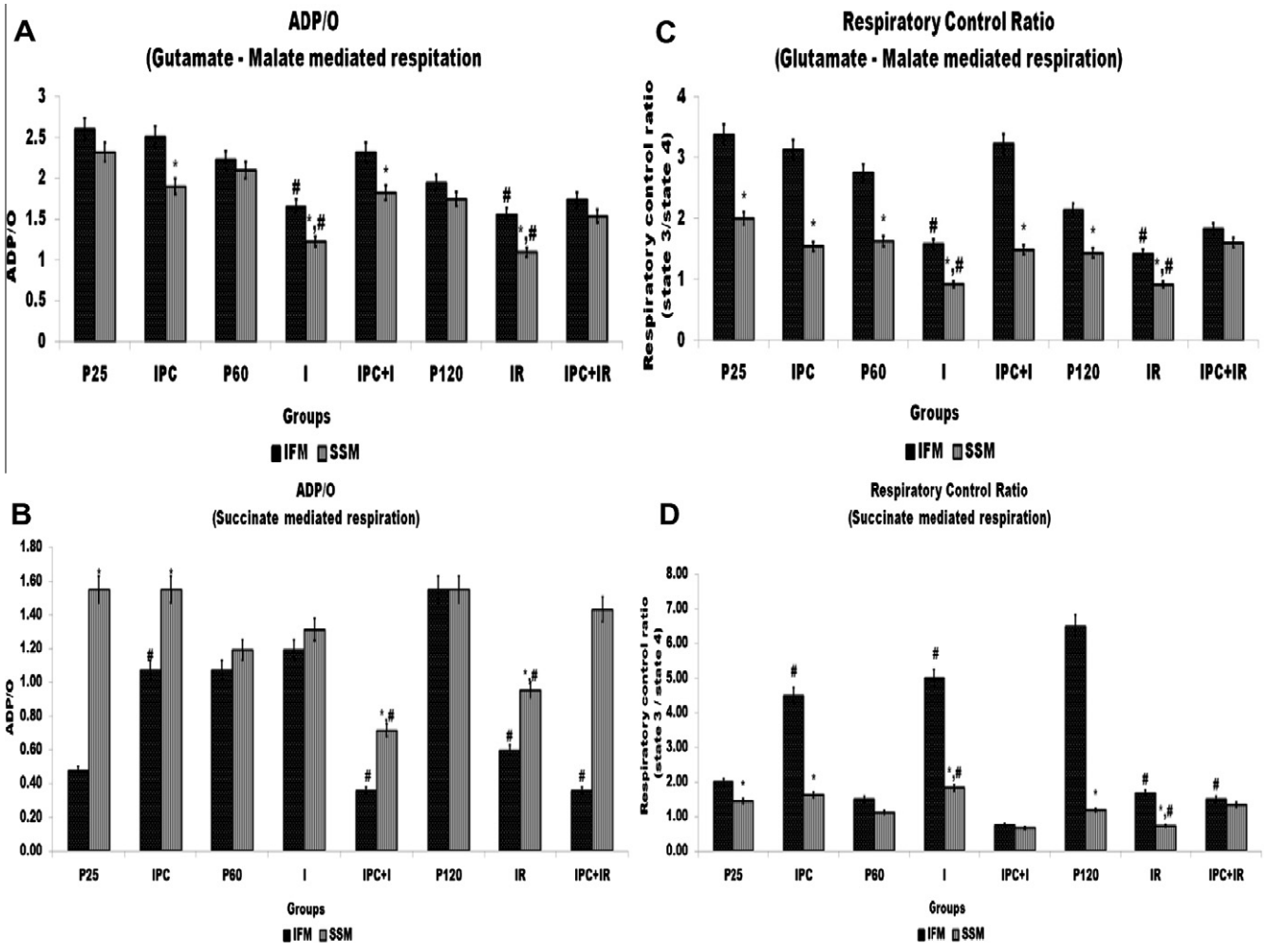

Fig. 1. Measurement of mitochondrial respiration of IFM and SSM in glutamate–malate (GM) medium and succinate medium. (A) ADP/O ratio in GM medium, (B) ADP/O ratio in succinate medium, (C) respiratory control ratio in GM medium and (D) respiratory control ratio in succinate medium. Results are expressed as mean ± SD of n = 4–6 independent assays. (∗) $p < 0.05$, statistically different from respective controls (P25 vs IPC; P60 vs I, IPC +I; P120 vs I/R, IPC +IR. (#) $p < 0.05$, statistically different between the sub populations.

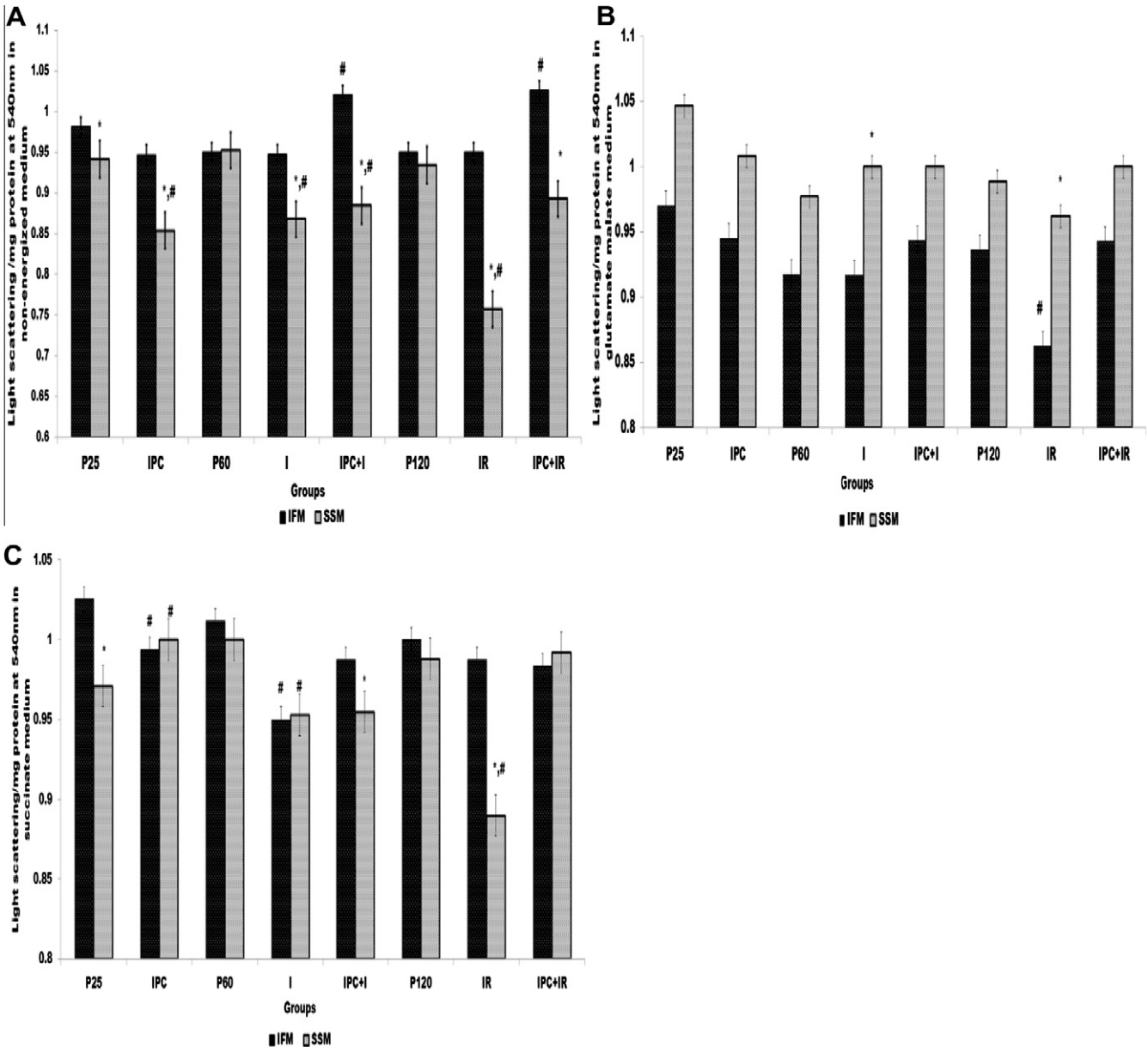

Fig. 2. Calcium induced mitochondrial swelling of IFM and SSM (A) non-energized medium, (B) glutamate malate medium and (C) succinate mediated medium. Results are expressed as mean ± SD of $n = 4–6$ independent assays: (∗) $p < 0.05$, Statistically different from respective controls (P25 vs IPC; P60 vs I, IPC +I; P120 vs I/R, IPC +IR. (#) $p < 0.05$, statistically different between the sub populations.

3.5. Determination of total glutathione concentration

A positive correlation between myocardial total glutathione content and the extent of ischemia reperfusion injury was reported earlier by Gupta et al. [20]. Significant increase in the total glutathione content of IFM was observed in the present study during ischemia and ischemia–reperfusion compared to P60 and P120 respectively (Fig 4). In contrast, relatively constant glutathione content was observed in SSM of the different experimental groups, compared to respective controls.

4. Discussion

The main information obtained from our experiments is that: (a) distinct oxidative phosphorylation capacities are exhibited in mitochondrial subpopulations during ischemia and reperfusion and are dependent on substrate specific respiration. For example, IFM showed better coupling capacity (both ADP/O ratio and RCR) when respiration was driven by the complex I substrate GM, while SSM exhibit better oxidative phosphorylation coupling (ADP/O ratio) than IFM with succinate as a respiration substrate. (b) Myocardial protection by ischemic preconditioning requires energized mitochondria.

4.1. Impact of ischemia, ischemia–reperfusion and ischemic preconditioning on oxidative phosphorylation of IFM and SSM

The present study shows that ischemia significantly reduced ADP/O and RCR in both IFM and SSM mitochondrial sub-populations only when respiration was driven by the complex I substrate GM, but not when it was driven by the complex II substrate succinate (Fig. 1). In fact, coupling efficiency (both ADP/O ratio and RCR) was increased in both sub-mitochondrial populations derived from ischemic hearts when respiration was driven by succinate. These

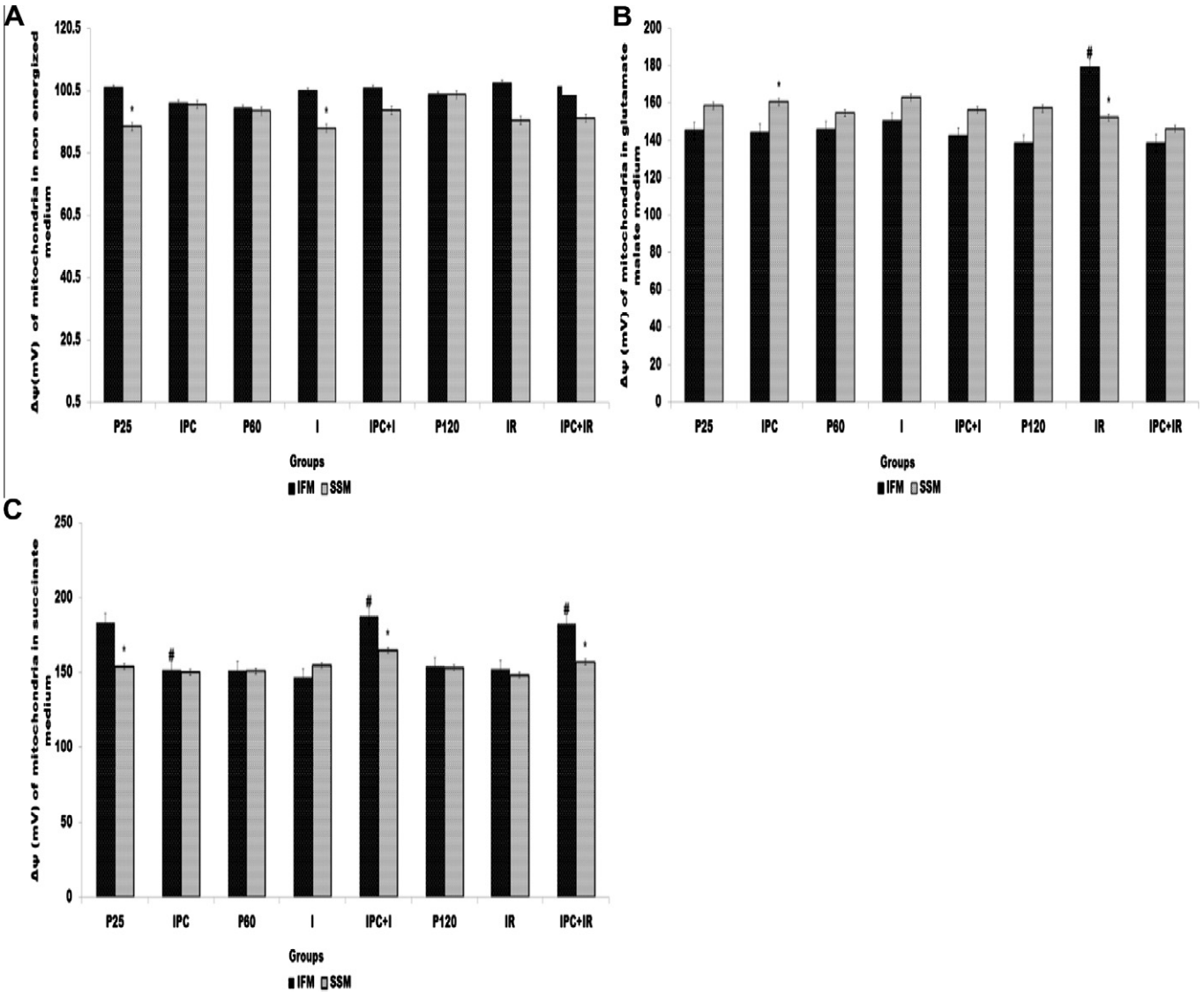

Fig. 3. Mitochondrial membrane potential of IFM and SSM. (A) non-energized medium, (B) glutamate malate medium and (C) succinate mediated medium. Results are expressed as mean ± SD of n = 4–6 independent assays. (∗) $p < 0.05$, Statistically different from respective controls (P25 vs IPC; P60 vs I, IPC +I; P120 vs I/R, IPC +IR. (#) $p < 0.05$, Statistically different between the sub populations.

results support the idea that during the ischemia complex II respiration prevails over the complex I respiration. This mutual inhibition between complex I and complex II was reported earlier [21] where it was shown that the increased activity of one of the complexes represses the activity of the other. This phenomenon can probably be attributed to competition between the complexes on ubiquinone [22], which might play an important role in sustaining mitochondrial function during ischemic conditions. The adverse effect of ischemia on respiratory coupling efficiency is more pronounced in SSM (Fig. 1A, 40% decline in ADP/O ratio in mitochondria isolated from ischemic hearts as compared to the P60 perfused hearts control) than in IFM (Fig. 1A, 20% decline in the same parameters). This difference is possibly due to the distinct impact of ischemia on mitochondrial sub-populations, possibly due to their different myocardial locations. Differences in oxidative phosphorylation between mitochondrial sub-populations respiring on GM or succinate were reported earlier in aging rats' hearts, where SSM showed lower state 3 respiration than IFM, in agreement with our observations [23].

Reperfusion of ischemic heart, reduced oxidative phosphorylation efficiency (ADP/O and RCR) in both IFM and SSM, whether GM or succinate was used as the substrate for respiration (Fig. 1). Thus the above mentioned inverse relationship between complex I and II respiration exhibited in mitochondrial sub populations during ischemia was not maintained in the reperfusion. As the inhibition of both ADP/O and RCR by I/R in IFM respiring on succinate was significantly more pronounced than the same inhibition in SSM respiring on succinate (Fig. 1), it is proposed that myocardial contractility under I/R conditions is supported by IFM mainly through the complex I respiration.

Prior preconditioning of ischemia and I/R rat heart brought the oxidative phosphorylation capacity of both IFM and SSM to near normal value in GM driven respiration. However, insuccinate meditated respiration, only SSM recovered significantly from the impact of ischemia and reperfusion (Fig. 1B and D). In I/R rats' hearts, both energized IFM and SSM showed significant swelling upon calcium administration when they were provided with the irrespective preferred substrates (IFMs well more than SSM in

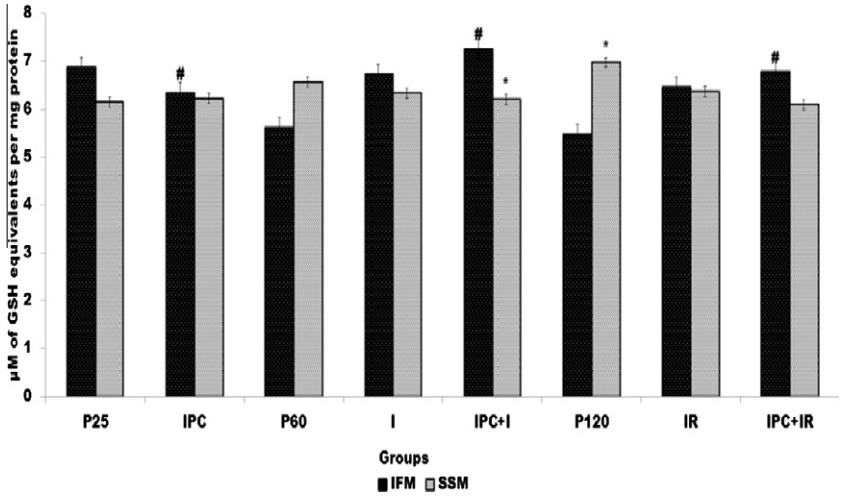

Fig. 4. Total glutathione level in IFM and SSM. Concentration is expressed as µM of GSH equivalents per mg proteins. Results are expressed as mean ± SD of $n = 4–6$ independent assays. (∗) $p < 0.05$, Statistically different from respective controls (P25 vs IPC; P60 vs I, IPC +I; P120 vs I/R, IPC +IR. (#) $p < 0.05$, Statistically different between the sub populations.

GM medium (Fig. 2B) and SSMs well more than IFM in succinate medium (Fig. 2C)). This indicates the possibility of surplus availability of calcium binding sites in IFM and SSM when they were energized with GM or succinate respectively Fig. 2C.

In contrast to energized mitochondria, non-energized mitochondrial subpopulations showed significant differences in their swelling pattern (Fig. 2A). SSM swelled more than IFM under both ischemia and ischemia–reperfusion. This phenomenon might be explained by Ca^{2+}-dependent swelling not involving specific Ca^{2+} binding sites and dependent on the redox state of mitochondria. The changes in the redox state of carriers of the mitochondrial respiratory chain correlated with the changes in the physiochemical function of the organelle and even with physiological parameters of the cardiac muscle [24]. Normal cells have to maintain specific redox couples, which include the relatively oxidized couple NAD^+/NADH and the relatively reduced couples $NADP^+$/NADPH and GSSG/2GSH, to maintain redox homeostasis. Our results show that mitochondrial GSH, one of the important bio-molecules regulating the oxidative stress, is declined in SSM as compared to IFM under ischemia and I/R conditions. Therefore reduced levels of total glutathione might lead to more ROS production which could open MPTP through oxidative damage and consequently decrease $\Delta\psi$ Thus higher SSM swelling in non-energized medium (Fig. 2A) may be due to the change in redox status of mitochondria (Fig. 4), indicating the possibility of calcium-dependent swelling.

Preconditioning the ischemic heart did not prevent swelling in SSM when they were not energized (Fig. 2A), indicates IPC is protective when mitochondria are energized. Conversely, IFM did show the protection against ischemic swelling upon ischemic preconditioning, even in non-energized state (Fig. 2A). This observation supports the notion that IFM and SSM have distinct biochemical natures, with higher ischemic resistance of IFM as compared to SSM [25].

4.2. Mitochondrial membrane potential in SSM and IFM

Based on our results, it has been shown that the membrane potential of isolated mitochondrial sub populations varies dependent upon its energy status. This difference might reflect the innate pathological inclination of the heart cells *in vivo*. Lower membrane potential could be a result of increased free radical generation which could compromise the integrity of the inner mitochondrial membrane via increased lipid peroxidation [26]. The observed elevated $\Delta\psi$ of IFM in non-energized and GM-energized conditions, compared to SSM, confirms the conclusions of a previous report on the ischemic resistant property of IFM [25]. Non-energized SSM showed reduced mitochondria membrane potential during ischemia and ischemia–reperfusion compared to its respective controls, but not in IFM. Thus we can predict that SSM may already be affected by ischemia and ischemia–reperfusion prior to IFM (further studies are needed). The reduced $\Delta\psi$ in SSM might indicate that their mitochondrial membrane has already compromised its integrity when subjected to ischemia and I/R. The increased calcium-induced swelling in SSM, caused by ischemia and ischemia–reperfusion in non-energized mitochondria (Fig. 2A) supports this assumption, as it can be explained by an increase in membrane permeability caused by oxidative damage. Based on the above observations we can conclude that when the heart mitochondria experiences an energy deficit, subpopulations of mitochondria like IFM and SSM behave physiologically different but do not compromise their primary functions. On the other hand, if the mitochondria are sufficiently energized with substrates, the sub populations behave similarly in a coherent fashion.

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