Opening of mitochondrial megachannel by iron: Competition of iron with calcium more important than oxidative stress

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INTRODUCTION

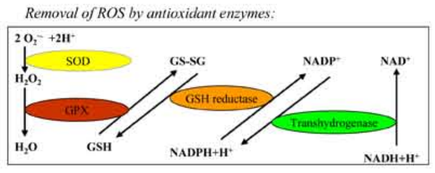
Mitochondrial permeability transition (MPT, also "mitochondrial megachannel") is an interesting phenomenon in which certain mitochondrial membrane proteins rearrange to create a giant pore, permeable for any substances up to 1,500 Da. The pore opening collapses the proton gradient of the inner membrane. MPT is induced by calcium and promoted by oxidative stress; it is classically inhibited by cyclosporin A or inhibitors of mitochondrial calcium import (Ruthenium Red). Transient 'flickering' of MPT pore plays a role in calcium signaling, while persistent MPT leads to mitochondrial swelling and cytochrome c release, implicated in necrotic and apoptotic cell death.

In vivo the oxidative stress is often catalyzed by iron. Most of the body iron is bound to proteins, only a small fraction of low-molecular-weight iron can generate oxidative stress as a donor of electrons in the Fenton reaction. Fe(II) ions are prone to autoxidation that also produces superoxide and hydrogen peroxide. Normally, antioxidant defense effectively protects from oxidative damage to biomolecules, but in case of e.g. iron overload it may become insufficient.

Reactive oxygen species (ROS) in the body:

1. Mitochondria or NADPH oxidase produce superoxide, $O_2^{-}\bullet$
2. Superoxide dismutates to hydrogen peroxide:
   $O_2^{-}\bullet + O_2^{-}\bullet + 2H^+ \rightarrow H_2O_2 + O_2$
3. The Fenton reaction: $H_2O_2 + Fe^{2+} \rightarrow OH^- + OH\bullet + Fe^{3+}$
4. Hydroxyl radical, OH• attacks biomolecules

Removal of ROS by antioxidant enzymes:

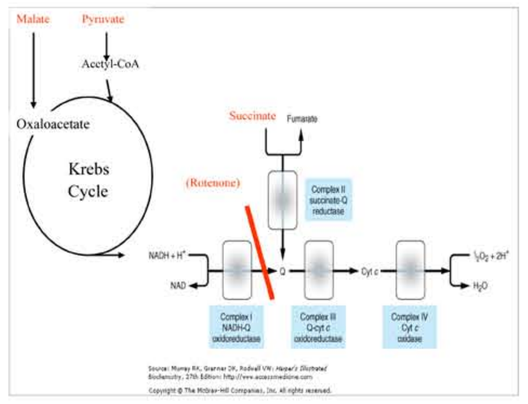


Ability of iron to induce MPT seems likely and indeed has been described in cultured hepatocytes, but mechanistic aspects of MPT induction by iron are poorly understood. According to literature, Fe(II) overloads antioxidant defense that shifts NAD(P)H/NAD(P) to oxidation, and the loss of reduced NAD(P)H promotes MPT opening. Iron can also inhibit the calcium uniporter; or can be imported by it to the mitochondrial matrix.

In this study we investigated the mechanism of MPT induction in isolated rat liver mitochondria by physiologically relevant (micromolar) concentrations of Fe(II) ions.

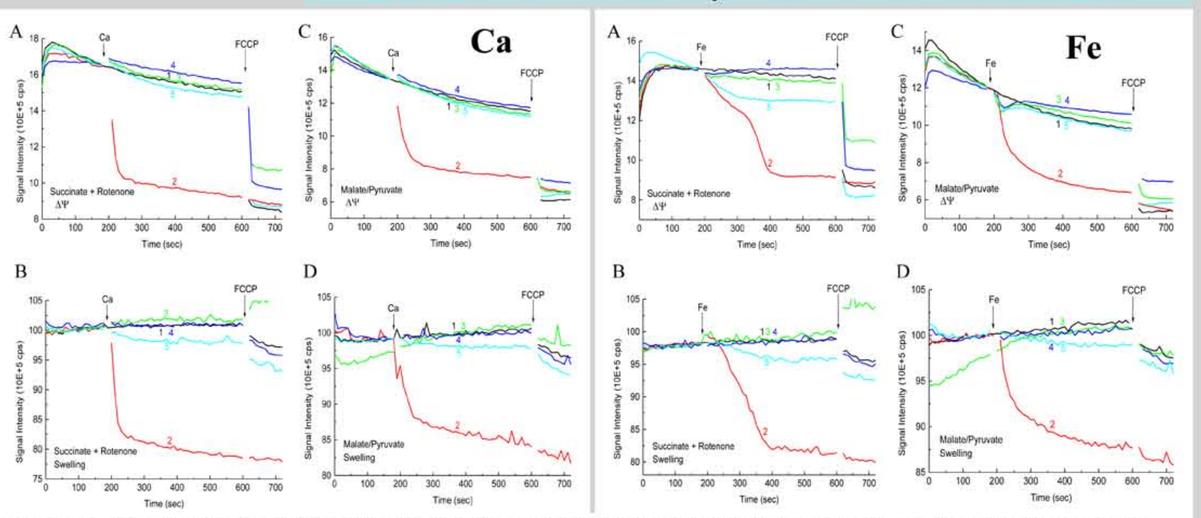
METHODS

- **Mitochondria isolated from rat liver** in 10 mM Tris/HCl buffer pH 7.4 containing 250 mM sucrose and 0.5 mM EDTA. Stabilized by addition of **bovine serum albumin**
- **Recording medium:** sucrose/KCl, buffered to pH 7.3, 1 mM phosphate, 37 ºC
- **Energization:** 5 mM sodium succinate (Complex III substrate, + 1 μM rotenone) or 2.5 mM potassium malate with 5 mM sodium pyruvate (Complex I substrates).
- **Measurements:**
  - **Mitochondrial swelling** (decrease of light scatter at 504 nm)
  - **The inner membrane potential** (fluorescent probe JC-1, 488/593 nm)
  - **NAD(P)H oxidation** (autofluorescence 340/465 nm)
  - **Concentration of iron** remaining in the medium (colorimetric assay with FerroZine and ascorbate)
  - **Concentration of calcium** in the medium (calcium ion-selective electrode, also fluorometry with Calcium Green-5N)
- **MPT induced by:**
  - **20 μM $CaCl_2$** (supposedly 7.5 μM Ca^{2+} over 12.5 μM of the residual EDTA)
  - **20 μM $FeCl_2$** (supposedly 7.5 μM Fe^{2+} over 12.5 μM of the residual EDTA)



RESULTS

1. MPT can be induced by micromolar Ca as well as Fe.

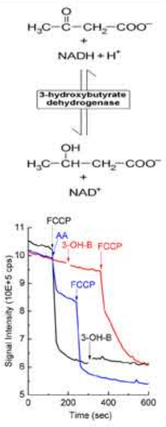


Mitochondria were energized by 5 mM succinate in the presence of 1 μM rotenone (**A, B**) or by 2.5 mM malate with 5 mM pyruvate (**C,D**). The potential of inner membrane (Δψ) was measured using the fluorescent probe JC-1 (**A, C**); simultaneously light scatter was recorded as the measure of mitochondrial swelling (**B, D**). **Trace 1** is always control measurement (no calcium or no iron), to **traces 2-5** 20 μM $CaCl_2$ or 20 μM $FeCl_2$ was added. MPT induced by calcium or iron is shown in **trace 2**, the inhibitory effect of excess (0.5125 mM) of EDTA is seen in **trace 3**, 2 μM Ruthenium Red in **trace 4**, and 5 μM cyclosporin A in **trace 5**. 5 μM FCCP was added before the end of each measurement as indicated.

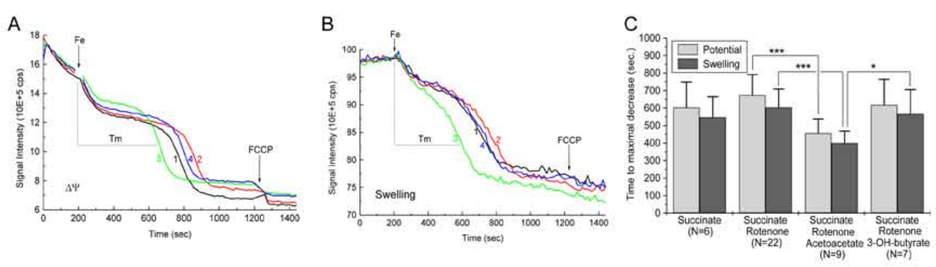
Hypothesis I: Iron redox cycling/autoxidation generates ROS, whose removal by antioxidant defense ultimately consumes electrons from NADH. Resulting drop in NADH/NAD ratio then promotes MPT opening

(A. Masini, T. Trenti, D. Ceccarelli, E. Ventura, The effect of ferric iron complex on isolated rat liver mitochondria. III. Mechanistic aspects of iron-induced calcium efflux, Biochim. Biophys. Acta 891 (1987) 150-156.)

2. Manipulation of NAD(P)H/NAD(P) ratio with 'ketone bodies' has only limited effect on iron-induced MPT.



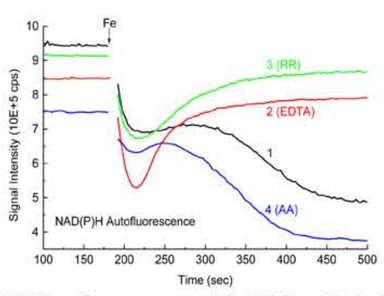
Autofluorescence (340/465 nm) of mitochondria respiring on succinate without rotenone measured following additions of 0.5 μM FCCP, 1 mM acetoacetate (AA), or 1 mM R-(−)-3-hydroxybutyrate (3-OH-B).



Mitochondria were energized by 5 mM succinate. The potential of inner membrane (Δψ) was measured using the fluorescent probe JC-1 (**A**); simultaneously light scatter was recorded as the measure of mitochondrial swelling (**B**). **Trace 1** is succinate only, in **traces 2-4** 1 μM rotenone was also present, **trace 3** is with addition of 1 mM acetoacetate, **trace 4** is with 1 mM R-(−)-3-hydroxybutyrate. 20 μM $FeCl_2$ and 5 μM FCCP were added to each mixture as indicated. Time to maximal decrease (Tm, shown only for trace 3) was read for each trace from its first derivative. **Graphs A & B** are representative traces for each condition. **Graph C** is a summary of all Tm data collected from measurements with three mitochondrial preparations. Mean ±SD is shown for each condition. ANOVA with Tukey-Kramer post-test was used for statistical evaluation. * …p<0.05; *** …p<0.001.

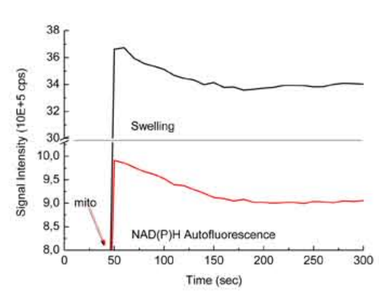
If iron induces MPT through NAD(P) oxidation, any experimental manipulation with the mitochondrial NADH/NAD⁺ ratio should affect the Fe-induced MPT as well. Surprisingly, omission of rotenone from the reaction mixture, or additions of the 'ketone bodies' had no pronounced effects on the course of MPT induced by iron. A series of repetitive measurements followed by quantification and statistical evaluation revealed that acetoacetate did accelerate the MPT induction, while omission of rotenone or addition of 3-hydroxybutyrate proved ineffective. The effect of 3-hydroxybutyrate, in fact, should be regarded as variable, as it inhibited Fe-induced MPT in some other measurements.

3. Iron induces biphasic NAD(P)H oxidation



The NAD(P)H autofluorescence was recorded at 340/465 nm. Mitochondria were energized by 5 mM succinate in the presence of 1 μM rotenone. 20 μM $FeCl_2$ was added to each mixture. **Trace 1:** effect of iron without further additions; **trace 2:** 512.5 μM EDTA; **trace 3:** 2 μM Ruthenium Red; **trace 4:** 1 mM acetoacetate.

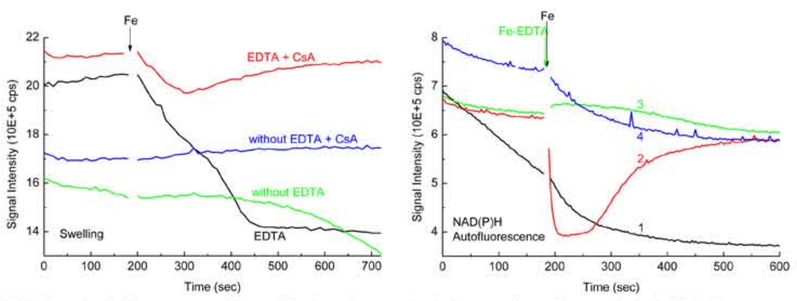
4. Delayed drop in NADH autofluorescence is only reflection of mitochondrial swelling



During energization both the light scatter and the NAD(P)H autofluorescence signal of the mitochondrial suspension decrease in parallel although the NAD(P)H should be fully reduced in the presence of rotenone.

It proves that changes in light scatter (here due to matrix volume changes during energization) interfere with the autofluorescence measurement.

5. Both iron-induced MPT and NAD(P)H oxidation occur only in the presence of residual EDTA.

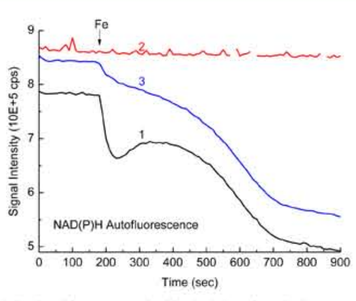


EDTA-free mitochondria were prepared by centrifugation and resuspension in the same volume of homogenization buffer lacking EDTA but still containing BSA; they were kept on ice and used within 120 minutes. The mitochondria were energized by 5 mM succinate in the presence of 1 μM rotenone. To each mixture, 20 μM $FeCl_2$ was added as indicated.

Left: Swelling was determined from the light scatter. The effect of iron on the EDTA-free mitochondria and the control mitochondria that still contained EDTA (12.5 μM) was considered. Where indicated, 5 μM cyclosporin A was also present.

Right: NAD(P)H oxidation determined from the 340/465 nm autofluorescence of EDTA-free mitochondria. **Trace 1:** effect of 20 μM $FeCl_2$ without further additions; **trace 2:** EDTA-free mitochondria diluted in a mixture containing 500 μM EDTA; **trace 3:** EDTA-free mitochondria diluted in a mixture containing 400 μM EDTA followed by the addition of a pre-mixed complex of 20 μM iron and 100 μM EDTA; **trace 4:** 5 μM cyclosporin A.

6. Catalase prevents iron-induced NAD(P)H oxidation but not MPT.



Mitochondria were energized by 5 mM succinate in the presence of 1 μM rotenone. NAD(P)H oxidation was determined from the 340/465 nm autofluorescence. **Trace 1:** effect of 20 μM $FeCl_2$ without further additions; **trace 2:** 33.3 μg/ml (333-1,332 U/ml) catalase present and no iron added; **trace 3:** 33.3 μg/ml catalase present and 20 μM $FeCl_2$ added as indicated.

EDTA chelates both FeII and FeIII, but the latter with much higher affinity and hence promotes rapid autoxidation of any bound FeII to FeIII. Our data indicate that it is the resulting burst of hydrogen peroxide what transiently oxidizes NAD(P)H. However, as catalase prevents NAD(P)H oxidation but not MPT induction, the iron-induced MPT is not caused by this NAD(P)H oxidation and the requirement for residual EDTA must be explained otherwise.

Hypothesis II: Iron that is not bound by EDTA is imported to mitochondrial matrix by the calcium uniporter and then induces MPT from within the mitochondria.

I.Romslo, T.Flatmark, Energy-dependent accumulation of iron by isolated rat liver mitochondria. II.Relationship to the active transport of Ca^{2+}, Biochim.Biophys.Acta 325 (1973) 38-46.

V. Gogvadze, P.B. Walter, B.N. Ames, Fe^{2+} induces a transient Ca^{2+} release from rat liver mitochondria, Arch. Biochem. Biophys. 398 (2002) 198-202.

7. Some iron is imported to mitochondrial matrix by calcium uniporter.

	Fe detected (μM)			
	−FCCP	**+FCCP**	**Mean difference**	**Paired t-test**
−RR	18.75±0.28	19.49±0.55	0.73±0.66	*
+RR	17.67±0.56	17.87±0.57	0.20±0.30	NS

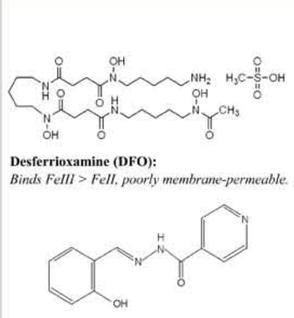
The isolated mitochondria were pre-mixed with 5 μM cyclosporin A, diluted to the recording medium in spectrofluorimetric cuvette as for the potential/light scatter measurements (DMSO instead of the JC-1 probe) containing 5 mM succinate + 1 μM rotenone, and challenged with 20 μM of $FeCl_2$ in the presence or absence of 5 μM FCCP and 2 μM Ruthenium Red (RR) as indicated.

After 5 minutes of incubation with iron the mixture was immediately centrifuged and supernatant used for colorimetric estimation of total remaining iron.

Mean ±SD from six estimations, obtained from two independent mitochondrial preparations. Each measurement without FCCP was followed by the corresponding mixture with FCCP, these values were paired and used for calculation of the mean difference. Paired t-test was used for statistical evaluation. * …p<0.05, NS … not significant.

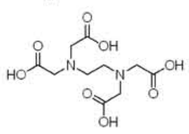
Ruthenium Red-sensitive Fe import: **0.5 μM (2 nmol Fe/mg mito protein)**

8. Post-addition of a membrane-permeable, but not impermeable iron chelator can prevent MPT induction by iron – but not always, whereas EDTA always effective.

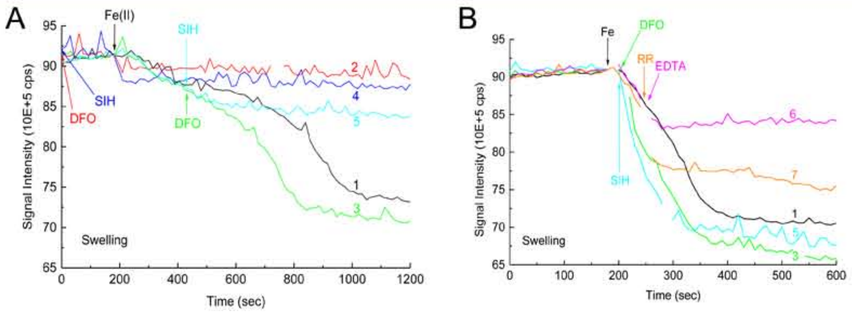


Desferrioxamine (DFO):
Binds FeIII > FeII, poorly membrane-permeable.

Salicylaldehyde isonicotinoyl hydrazone (SIH)*:
Binds FeIII > FeII, lipophilic, very effectively penetrates through membranes.



Ethylenediaminetetraacetic acid (EDTA):
Binds FeIII > FeII (also Ca, Mg, etc.), hydrophilic, membrane-impermeable.



Mitochondria were energized by 5 mM succinate in the presence of 1 μM rotenone. 20 μM $FeCl_2$ was added to each mixture as indicated. **A:** An experiment where iron-induced MPT was slow; chelators desferrioxamine (DFO) or salicylaldehyde isonicotinoyl hydrazone (SIH) were either present from the beginning, or added 4 minutes after iron. Swelling measured as light scatter. **B:** Another experiment where iron-induced MPT was relatively fast (note the different time scale), effects of post-additions of DFO, SIH, EDTA and Ruthenium Red are shown. Swelling measured as light scatter. **Trace 1** always represents effect of 20 μM $FeCl_2$ without further additions; **trace 2** (only A): 50 μM DFO before iron; **trace 3:** 50 μM (A) or 100 μM (B) of DFO added after iron as indicated; **trace 4** (only A): 50 μM SIH before iron; **trace 5:** 50 μM (A) or 100 μM (B) of SIH added after iron as indicated; **trace 6**: 500 μM EDTA added after iron as indicated; **trace 7**: 2 μM Ruthenium Red added after iron as indicated.

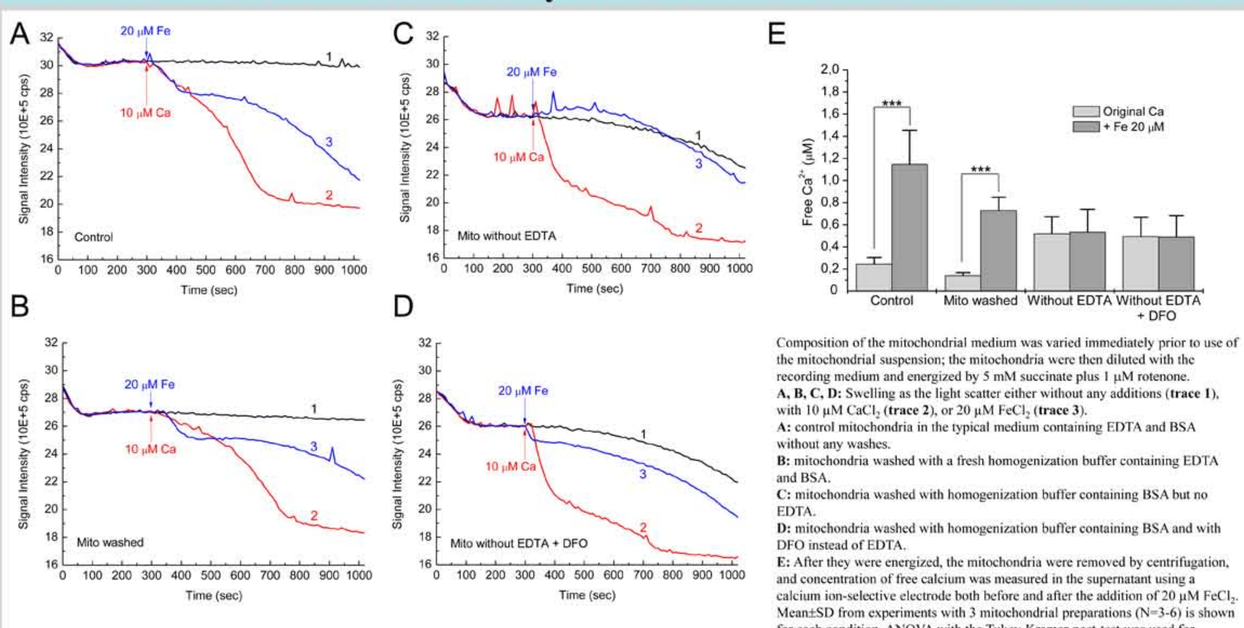
In experiments where iron-induced MPT was rather slow (graph A), only SIH, but not DFO, prevented the second delayed phase of iron-induced MPT when added several minutes after iron, which is exactly what should be expected if iron induces MPT inside the mitochondria. However, there were other experiments where MPT was quite rapid (graph B) and then the SIH, even when added as early as 20 seconds after the iron, consistently accelerated the swelling instead of inhibiting it. Both DFO and SIH could accelerate MPT because of NAD(P)H oxidation – the chelators are similar to EDTA in preferential binding of FeIII over FeII, which enhances autoxidation of any bound FeII.

On the other hand, addition of EDTA or Ruthenium Red was always able to stop the iron-induced MPT, no matter whether added before the iron, or after. Even if MPT is started by iron, from certain point of time the phenomenon propagates by calcium cycling and iron is no longer necessary.

*The SIH chelator was a kind gift from prof. Prem Ponka, McGill University, Montreal.

Hypothesis III: EDTA acts as a reservoir of endogenous/adventitious calcium ions that can be displaced by iron.

9. Mitochondria are sensitive to iron only in the medium in which iron liberates calcium ions



Composition of the mitochondrial medium was varied immediately prior to use of the mitochondrial suspension; the mitochondria were then diluted with the recording medium and energized by 5 mM succinate plus 1 μM rotenone.

A, B, C, D: Swelling as the light scatter either without any additions (**trace 1**), with 10 μM $CaCl_2$ (**trace 2**), or 20 μM $FeCl_2$ (**trace 3**).

A: control mitochondria in the typical medium containing EDTA and BSA without any washes.

B: mitochondria washed with a fresh homogenization buffer containing EDTA and BSA.

C: mitochondria washed with homogenization buffer containing BSA but no EDTA.

D: mitochondria washed with homogenization buffer containing BSA and with DFO instead of EDTA.

E: After they were energized, the mitochondria were removed by centrifugation, and concentration of free calcium was measured in the supernatant using a calcium ion-selective electrode both before and after the addition of 20 μM $FeCl_2$. Mean±SD from experiments with 3 mitochondrial preparations (N=3-6) is shown for each condition. ANOVA with the Tukey-Kramer post-test was used for statistical evaluation. ***p<0.001.

CONCLUSION

In our experiments, 20 μM ferrous chloride was sufficient to trigger the MPT in isolated mitochondria respiring either on succinate with rotenone or on malate/pyruvate. The potency of iron with respect to MPT induction was comparable to that of calcium, and the classical features of the MPT, such as inhibition by cyclosporin A or by blocking the calcium uniporter, could be demonstrated with both metals. Interestingly, although the general chelator EDTA inhibited MPT when in excess, some EDTA was required in the reaction mixture for the iron to be effective.

Two mechanisms were initially considered based on the evidence in the literature: 1) mitochondrial NAD(P)H oxidation due to oxidative stress produced by iron, and 2) iron uptake into the mitochondrial matrix by the calcium uniporter. Both events occur in our experimental system, but they are only marginally involved in the iron-induced MPT. The primary mechanism observed in our experiments was the displacement of adventitious/endogenous calcium from the residual EDTA by iron.

Although artificially created, this interplay between iron and calcium could be considered as an important and probably fairly general mechanism of iron toxicity in the cells because:

- Both concentrations of 'free' iron (7.5 μM) and calcium (0.13 μM) in our experiments were close to physiological situation.
- Divalent metal ions are similar – many calcium chelators bind also Zn, FeII (the opposite is not true).
- Cytosol has a high ability to buffer calcium ions. Although there is no EDTA in the cells, a medium containing Ca-EDTA can be closer to in vivo situation than simple buffers.
- Cytosol is also highly reducing, meaning that any 'free' iron is likely to exist in the soluble reduced FeII form.
- Concentration of oxygen inside the cells is 100-300-fold lower than in the outer world where the in vitro experiments are performed. Therefore, inside the cells FeII is more likely to stay reduced and compete for binding sites with calcium ions, rather than undergo redox cycling generating oxygen radicals.

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