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THE EFFECT OF FERRIC IRON COMPLEX ON Ca^{2+} TRANSPORT IN ISOLATED RAT LIVER MITOCHONDRIA

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SUMMARY: The in vitro effects of iron (III)-gluconate complex on the production of malondialdehyde and on the Ca^{2+} transport in isolated rat liver mitochondria were studied. A correlation between the concentration of iron added and the formation of malondialdehyde was found. The enhancement by iron of lipid peroxidative process in the mitochondrial membrane brought about the induction of Ca^{2+} release from mitochondria. Experimental evidence based on the membrane potential pattern of mitochondria pre-loaded with a low pulse of Ca^{2+} suggested that Ca^{2+} efflux was not due to a nonspecific increase in the inner membrane permeability, i.e. to a collapse of membrane potential, but rather to the activation of an apparently selective pathway for Ca^{2+} release.

The mechanism of hepatocellular injury in chronic iron overload, i.e. the role that iron plays in the pathogenesis of liver cell damage, has not yet been experimentally established (1). Peroxidative damage to the lipid membranes of cellular organelles resulting in functional and structural alterations in cell integrity may constitute a plausible explanation (1). Indeed, evidence is accumulating that iron can enhance lipid peroxidation not only in vitro (2-5) but also in vivo (6-10) in liver mitochondria. Lipoperoxidation may modify the fluidity state of the bilayer phospholipids (11), thus in-

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid; BHT, butylated hydroxytoluene; Trolox-C, 2,5, 7,8 tetramethyl-6-chromanol; MDA, malondialdehyde; FCPP, carbonylcyanide-p-trifluorometoxyphenylhydrazone; $\Delta\psi$, mitochondrial transmembrane electrical potential, negative inside.

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fluencing the transport of solutes and ions in mitochondria. Liver mitochondria isolated from iron treated rats displayed anomalies in the inner membrane properties, i.e. they presented an anomalous transmembrane potential (9,10,12). Relevant to this point are the observations that the in vitro induction of peroxidation reactions in mitochondrial membranes by hydroperoxides brings about the induction of K^+ efflux (13). The activation of Ca^{2+} release process in liver mitochondria by hydroperoxides was also reported (14,15).

In the present paper the effect of iron(III)-gluconate complex on the susceptibility of the mitochondrial membrane to lipid peroxidation was studied. The process of mitochondrial Ca^{2+} transport under these experimental conditions was parallely investigated.

MATERIALS AND METHODS

Rat liver mitochondria were prepared in 0.25 M sucrose according to a standard procedure (16).

The amount of malondialdehyde (MDA) formed was measured by the thiobarbituric acid method (17) in the presence of 0.1% butylated hydroxytoluene (BHT) and 0.5 mM $FeCl_3$. Mitochondria (1mg/ml) were incubated under State 4 conditions at 37°C. The incubation medium was as follows: 120 mM KCl; 10 mM Hepes (pH 7.4) and 2.5 mM potassium succinate as the substrate. Samples of 1 ml were collected at specified times and the reaction stopped by rapid mixing with 2 ml of thiobarbituric acid mixture. The amount of MDA was determined in the supernatant, after centrifugation, spectrophotometrically at 535 nm.

The transmembrane potential ($\Delta\psi$) was measured by monitoring with a tetraphenylphosphonium selective electrode the movements of tetraphenylphosphonium across the mitochondrial membrane as in (18). Mitochondria (3mg/ml) were incubated at 25°C in 210 mM mannitol; 70 mM sucrose; 10 mM Hepes (pH 7.4); 5 μM rotenone and energized by 2.5 mM potassium succinate.

Ca^{2+} movements were followed at 25°C by a Ca^{2+} selective electrode as described in (19). The incubation medium contained 120 mM KCl; 10 mM Hepes (pH 7.4); 3 mg/ml of mitochondrial protein; 5 μM rotenone and 50 μM $CaCl_2$. After a preincubation period of 5 min the initial rate of Ca^{2+} uptake was measured following the addition of 5 mM potassium succinate.

Protein was determined by a biuret method with bovine serum albumin as standard.

Iron(III)-gluconate (Ferlixit) was purchased from Natter-mann and Cie, Koln (F.R.G.). Trolox-C was gift from Roche Italia, Milano (Italy).

RESULTS AND DISCUSSION

The effect of iron(III)-gluconate at various concentrations on the malondialdehyde production in isolated rat liver mitochondria is presented in Fig. 1. It appears that iron induces MDA formation, to an extent which is proportional to the concentration of iron added, in mitochondria respiring under State 4 conditions. Concentrations of iron as low as 100 μM are able to elicit a significant enhancement of lipid peroxidation process. When antioxidant agents such as BHT and Trolox-C were present in the reaction medium they completely prevented MDA formation (not shown).

Fig. 2 shows the effect of iron complex on the membrane potential of liver mitochondria during the transport of a low pulse of external Ca^{2+}. Energization of mitochondria with succinate results in the development of a membrane potential (negative inside) of about 180 mV. The addition of a low pulse of

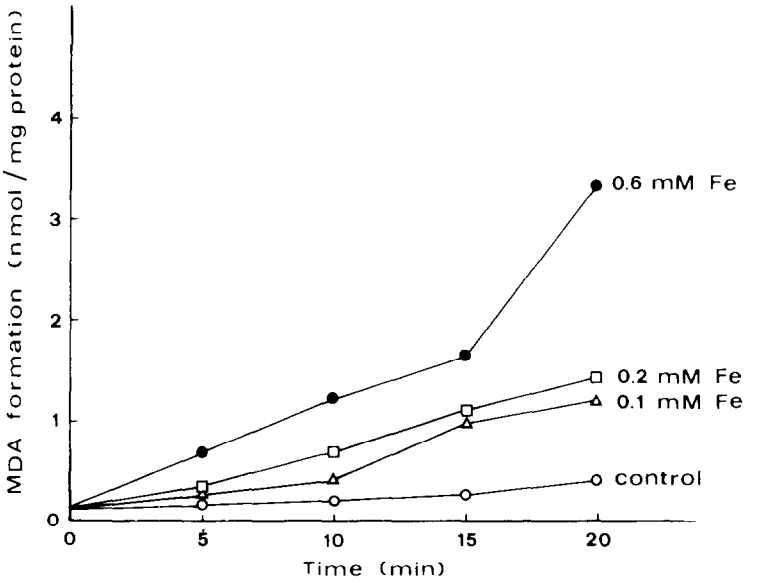

Fig. 1 Malondialdehyde formation in rat liver mitochondria induced by various concentrations of iron(III)-gluconate. Mitochondria were incubated under State 4 conditions at 37°C and MDA was determined, as described in the Methods, in the absence and in the presence of various concentrations of iron(III)-gluconate (Fe). The reaction started by the addition of mitochondria.

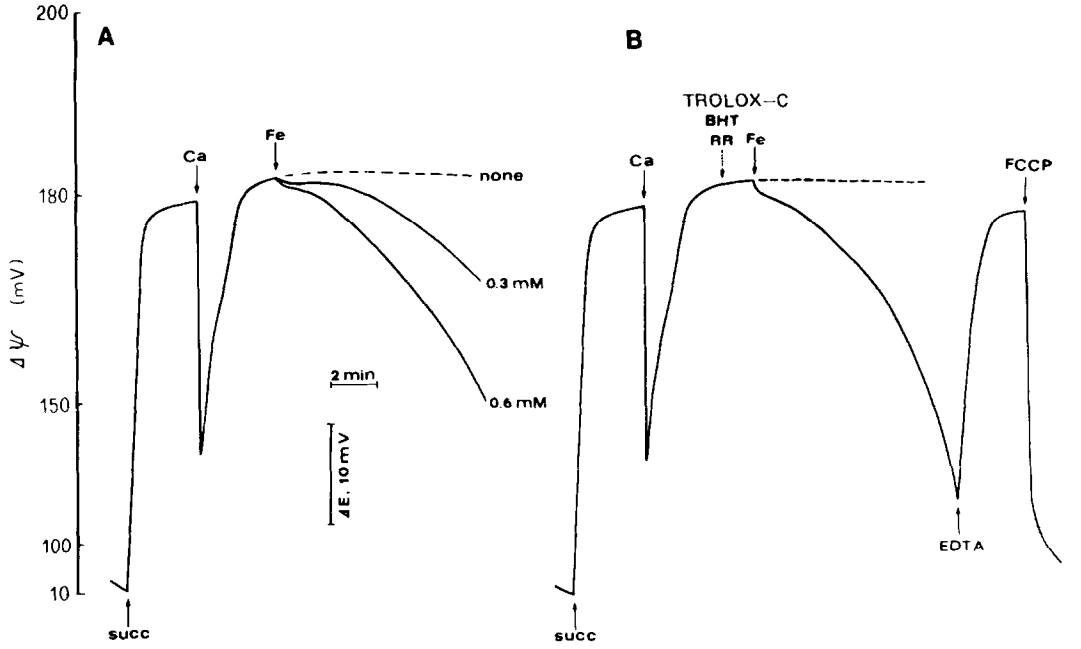

Fig. 2 The effect of iron(III)-gluconate on the transmembrane potential of rat liver mitochondria pre-loaded with a low pulse of Ca^{2+}. Mitochondria in standard incubation medium, described in the Methods, were energized by addition of 2.5 mM succinate (succ). Arrows indicate the following additions: 100 μM Ca^{2+}; iron(III)-gluconate (Fe); 30 μM butylated hydroxytoluene (BHT); 200 μM Trolox-C; 1 μM ruthenium red (RR); 0.5 mM EDTA; 1 μM FCCP. The concentration of Fe added in Fig. 2B was 0.6 mM. The dashed line in Fig. 2B indicates the membrane potential trace when either Trolox-C, BHT or RR where present before the addition of Fe. The transmembrane potential ($\Delta\psi$) was measured as described in the Methods in the presence of 15 μM tetraphenylphosphonium chloride. ΔE, electrode potential.

Ca^{2+} (30 nmol/mg protein) induces a sudden decrease in $\Delta\psi$. When this pulse of Ca^{2+} has been accumulated, $\Delta\psi$ trace reverses itself spontaneously and a new steady-state almost identical to pre-Ca^{2+} level is reached. Addition of iron, after a lag period, induces a progressive drop in membrane potential (Fig. 2A). Either the lenght of the lag phase or the rate of $\Delta\psi$ drop are closely dependent on the iron concentration. This pattern of $\Delta\psi$ trace may be reasonably due to the re-uptake of Ca^{2+} released as a consequence of the induction of lipoperoxidation reaction catalyzed by iron in the mitochondrial membrane, i.e. to the enhancement of Ca^{2+} cycling. The involvement of lipoperoxidation of mitochondrial membrane lipids in the Ca^{2+} relea-

sing process is directly demonstrated by the results presented in Fig. 2B. Indeed, it is seen that addition of antioxidant agents such as BHT and Trolox-C, at concentrations which inhibit lipid peroxidation induced by iron, prevents the membrane potential fall.

The results presented in Fig. 2B seem also to indicate that the effect of the lipoperoxidation process by iron complex on the mitochondrial inner membrane is rather selective for Ca^{2+} transport. Indeed, the activation of the release route for Ca^{2+} seems to result from a change of the membrane ambience around the Ca^{2+} release system rather than from a nonspecific increase in the permeability of the inner membrane. In the latter case, the collapse of the membrane potential would be the early event thus leading to the Ca^{2+} release by reverse activity of the uniporter. Two lines of experimental evidence seem to support the former proposal. Firstly, addition of ruthenium red, a specific inhibitor of the electrophoretic Ca^{2+} uptake to inhibit the reuptake of Ca^{2+}, to mitochondria pre-loaded with a low pulse of Ca^{2+}, prevents the drop of $\Delta\psi$ induced by iron, so indicating that the enhancement of Ca^{2+} cycling is responsible for the fall of $\Delta\psi$. Secondly, addition of EDTA, to limit the operation of Ca^{2+} cycling by lowering the extramitochondrial Ca^{2+} and thus inhibiting its reuptake, when the membrane potential has reached the lowest steady-state in the presence of iron, induces a rapid and almost complete reversal of the membrane potential trace to the pre-Ca^{2+} steady-state level. This indicates that the inner membrane is not structurally damaged under these experimental conditions and that mitochondria exhibit a normal membrane potential when the dissipation of energy due to the reuptake of Ca^{2+} released is blocked.

Direct experimental evidence on the effect of iron(III)-gluconate complex on the mitochondrial calcium transport are presented in Fig. 3, where Ca^{2+} fluxes were monitored with a Ca^{2+} selective electrode. It is seen that addition of iron after completion of Ca^{2+} uptake, results in an efflux of Ca^{2+} follo-

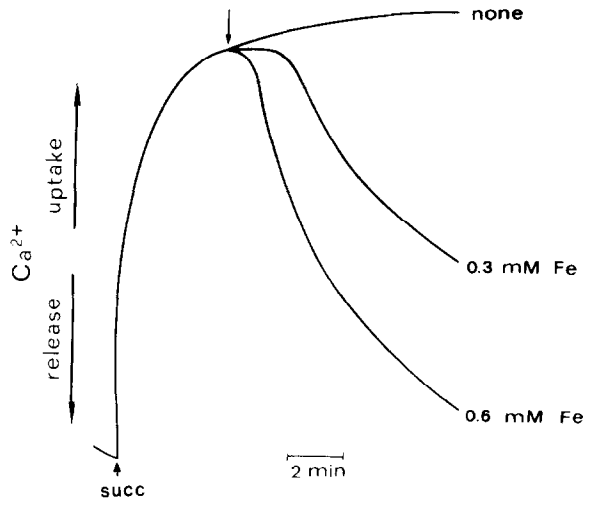

Fig. 3 The effect of iron(III)-gluconate on the Ca^{2+} fluxes of rat liver mitochondria. Mitochondria were incubated for 5 min in the standard reaction medium described in the Methods in the presence of 50 μM Ca^{2+}. After the efflux of endogenous Ca^{2+} had ceased, the accumulation of Ca^{2+} was started by the addition of 5 mM succinate (succ). When present, iron(III)-gluconate (Fe) was added at the point indicated by the arrow. Ca^{2+} movements were monitored by a Ca^{2+} selective electrode descibed in the Methods.

wing a brief lag period. The lenght of the lag phase and the rate of the Ca^{2+} efflux are dependent on the concentration of iron added.

The present results indicate a correlation between induction of lipoperoxidative process in the mitochondrial membrane and alteration in the mitochondrial Ca^{2+} transport. Indeed the formation of MDA in isolated rat liver mitochondria following the addition of a ferric iron complex, such as iron(III)-gluconate, here reported for the first time, is accompanied by alterations in the mitochondrial membrane resulting in Ca^{2+} efflux from mitochondria. The experimental evidence here presented suggests that this situation does not reflect gross membrane damages but rather subtle modifications which bring about the activation of an apparently selective pathway for Ca^{2+} release.

The *in vitro* effects of ferric iron complex on the mitochondrial Ca^{2+} transport appear of interest for the comprehension of the mechanisms of hepatocellular injury in chronic iron over-

load. Indeed, given the basic role that mitochondria play in the intracellular Ca^{2+} homeostasis, an alteration of mitochondrial Ca^{2+} transport may conceivably be a factor in the onset of the cell damage in this pathology.

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