Comparative effects of the Roundup and glyphosate on mitochondrial oxidative phosphorylation

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Abstract

The potential toxicity of the herbicide Roundup and its fundamental substance (glyphosate) was tested in bioenergetic functions of isolated rat liver mitochondria. Roundup stimulates succinate-supported respiration twice, with simultaneous collapse of transmembrane electrical potential, while glyphosate used in the same concentrations does not induce any significant effect. Additionally, Roundup depresses state 3 respiration by about 40%, at 15 mM, whereas uncoupled respiration in the presence of FCCP is depressed by about 50%. Depression of uncoupled respiratory activity is mediated through partial inhibition of mitochondrial complexes II and III, but not of complex IV. The phosphorylative system was affected by both a direct and an indirect effect on the F0F1 ATPase activity. The addition of uncoupled concentrations of Roundup to Ca²⁺-loaded mitochondria treated with Ruthenium Red resulted in non-specific membrane permeabilization, as evidenced by mitochondrial swelling in isosmotic sucrose medium. Therefore, the uncoupling of oxidative phosphorylation is also related to the non-specific membrane permeabilization induced by Roundup. Glyphosate alone does not show any relevant effect on the mitochondrial bioenergetics, in opposition to Roundup formulation products. The differences in the toxicity observed could be either attributed to some products of Roundup or to a synergic effect of glyphosate and formulation products. Bearing in mind that mitochondria is provided with a variety of bioenergetic functions mandatory for the regulation of intracellular aerobic energy production and electrolyte homeostasis, these results question the safety of Roundup on animal health.

Keywords: Roundup; Glyphosate; Mitochondrial bioenergetics; Oxidative phosphorylation; Mitochondrial permeability transition

1. Introduction

Glyphosate, N-(phosphonomethyl)glycine (Fig. 1), the active ingredient of very well-known herbicide preparations, such as Roundup, is a systemic and non-selective herbicide used to kill broadleaved grass, and sedge species (Williams et al., 2000). Although glyphosate is already one of the most used xenobiotics in modern agriculture, we should expect an increasing utilization of glyphosate largely due to the number of transgenic plants developed to be tolerant to this herbicide (May et al., 2002; Nadler-Hassar et al., 2004; Stephenson et al., 2004) and due to the removal of patent protection for Roundup by Monsanto Co. Glyphosate’s LD₅₀ in rats is greater than 4320 mg/kg of the body weight (EPA, 1993). The effects of glyphosate on hepatic and intestinal drug metabolizing enzyme activities studied in rats, intragastrically exposed for two weeks, revealed
that glyphosate decreased the hepatic level of cytochrome P-450 and monoxygenase activities as well as the intestinal activity of aryl hydrocarbon hydroxylase (Hietanen et al., 1983).

Although many studies have shown that glyphosate has a low toxicity (Marc et al., 2002; Howe et al., 2004; Pieniazek et al., 2004), Barbosa et al. (2001) reported that a man accidentally sprayed with glyphosate developed a symmetrical Parkinson syndrome. Some authors have reported an increased risk for non-Hodgkin’s lymphoma (NHL) following exposure to certain pesticides (e.g. Roundup) (Hardell et al., 2002; De Roos et al., 2003). Virtually, every pesticide product contains ingredients other than those identified as the “active” ingredient(s), i.e. the one designed to provide the killing action. These ingredients are misleadingly called “inert”. Commercial glyphosate formulations are more acutely toxic than glyphosate, since the amount of Roundup required to kill rats is about 1/3 of the amount of glyphosate alone (Martinez and Brown, 1991). Similar results have been obtained in cell division, thus indicating a synergy between glyphosate and Roundup formulation products (Marc et al., 2002).

Electron microscopy of fish (Cyprinus carpio) exposed by emersion in Roundup revealed that the herbicide caused the appearance of myelin-like structures in carp hepatocytes, the swelling of mitochondria and the disappearance of the internal membrane of mitochondria (Szarek et al., 2000).

The present study constitutes the very first attempt to describe the interaction of Roundup and glyphosate with mitochondrial functions. These studies are relevant to toxicology because most of the energy involved in cellular metabolism and intermediary metabolic compounds is generated at the expense of mitochondrial respiration (Erecinska and Wilson, 1982). Interactions of chemical compounds with mitochondrial functions can, therefore, result in severe impairment of the general metabolism.

2. Materials and methods

2.1. Isolation of rat liver mitochondria

Wistar rats (200–300 g) were starved overnight before being killed by cervical displacement. The isolation was performed by the conventional method (Gazzotti et al., 1997), with minor modifications. The homogenization medium contained 0.25 M sucrose, 5 mM HEPES (pH 7.4), 0.2 mM EGTA (ethyleneglycol-bis(β-aminoethyl ether) N,N’,N’’,N’’’-tetraacetic acid) and 0.1% fatty acid-free bovine serum albumin (BSA). EDTA (ethylene-diaminetetraacetic) EGTA and BSA were omitted from the final washing medium, which was adjusted to pH 7.2. The final concentration of the mitochondrial protein was determined by the biuret method (Gornall et al., 1949) using BSA as standard. The experiments reported here were carried out in accordance with the National Requirements for Vertebrate Animal Research and the European Convention for the Protection of Animals used for Experimental and other Scientific Purposes.

2.2. Mitochondrial respiratory activity

Oxygen consumption of isolated mitochondria was measured polarographically using a Clark-type oxygen electrode (Ernster and Kuylenstierna, 1970) connected to a suitable recorder in a closed water-jacketed 1.0 ml chamber with magnetic stirring, at 25 °C. Respiration rates were calculated assuming an oxygen concentration of 450 nAt O/ml in the experimental medium at 25 °C. The standard respiratory medium consisted of 130 mM sucrose, 50 mM KCl, 5 mM MgCl2, 5 mM KH2PO4, and 5 mM HEPES, pH 7.2. Roundup or glyphosate was added in aliquots (a few microlitres), from a concentrated aqueous solution (1 M, adjusted to pH 7.0), to 1 ml of the standard respiratory medium supplemented with mitochondria (0.8 mg protein) and allowed to incubate for 5 min before the addition of respiratory substrate, i.e., before the beginning of the respiratory activity. In all the experiments, Roundup (mM) concentrations are expressed in terms of the final glyphosate concentration presented in each assay. State 3 was elicited by adding adenosine 5’-diphosphate (ADP; 1 mM), and uncoupled respiration by adding 1 μM FCCP. The respiratory control ratio (RCR) and ADP to oxygen ratio (ADP/O) were calculated according to the method previously described (Chance and Williams, 1956), considering that the saturation oxygen concentration was 232 nmolO2/ml in the reaction medium at 25 °C.

2.3. Membrane potential (ΔΨ) measurements

The reactions were conducted under a continuous stream of O2 to avoid anaerobiosis. The transmembrane potential (ΔΨ) was estimated with a TPP+ electrode according to the equation of Kamo et al. (1979), without correction for the “passive” binding contribution of TPP+ to the mitochondrial membranes (as the purpose of the experiment was to show relative changes in the potential rather than absolute values). A matrix volume of 1.1 μl/mg mitochondrial protein was considered and
valinomycin was used to calibrate the basal line. Reactions were carried out at 25 °C in 1 ml of the standard respiratory medium (the same medium as described for the oxygen consumption experiments) supplemented with 3 μM TPP and 0.8 mg mitochondrial protein. Calibration runs in the presence of both xenobiotics excluded any direct interference in the electrode signal.

2.4. Enzymatic activities

Succinate dehydrogenase activity was measured spectrophotometrically by the reduction of DCIP (2,6-dichlorophenolindophenol) at 600 nm in the presence of PMS (phenazine methasulphate) (Singer, 1974). The reaction was performed in 1 ml of the standard reaction medium supplemented with 5 mM succinate, 2 μM rotenone, 0.1 μg antimycin A, 1 mM KCN, 0.025% Triton X-100 at 25 °C and 0.5 mg protein of disrupted mitochondria (two cycles of freezing and thawing).

Succinate cytochrome c reductase activity was measured spectrophotometrically (Tisdale, 1967) at 25 °C by following the reduction of oxidized cytochrome c by the increase in absorbance at 550 nm. The reaction was initiated by the addition of 5 mM succinate to 2 ml of the standard reaction medium supplemented with 2 μM rotenone, 1 mM KCN, 54 μM of cytochrome c and 1 mg protein of broken mitochondria.

Cytochrome c oxidase activity was measured polarographically (Brautigan et al., 1978) at 25 °C in 1 ml of the standard reaction medium supplemented with 5 mM succinate, 2 μM rotenone, 10 μM cytochrome c and 0.5 mg protein broken mitochondria. The reaction was initiated by the addition of 5 mM ascorbate plus 0.25 mM TMPD.

ATP-synthase activity was determined by monitoring the pH increase associated with ATP synthesis (Madeira et al., 1974). The reaction was carried out in 2 ml of the reaction medium containing 130 mM sucrose, 50 mM KCl, 5 mM MgCl2 and 2 mM KH2PO4 (pH 7.2), supplemented with 5 mM succinate and 0.5 mg of protein. The reaction was initiated by the addition of 200 μM ADP to the mitochondrial suspension. The pH change was evaluated with a Crison pH meter connected to a Perkin–Elmer recorder. The addition of oligomycin (2 μg/mg protein) completely halted the production of protons.

2.5. Mitochondrial swelling

Mitochondrial osmotic swelling was monitored by detecting turbidity, at 520 nm with a suitable spectrophotometer-recorder set up. The reactions were carried out at 25 °C in 2.0 ml of the required media as indicated in legends to figures.

2.6. Chemicals

All reagents were of analytical grade for research. Glyphosate was supplied by the Monsanto Company and Roundup was purchased in a specialized store.

2.7. Treatment of the data

Mean results are presented with the in respective ±SD values from at least three independent experiments. Statistical analyses were performed using two-tailed unpaired t-tests. A p value <0.05 was considered statistically significant.

3. Results

3.1. Xenobiotic effects on the mitochondrial respiration

The effects of Roundup and glyphosate on succinate-dependent respiratory indexes, RCR and ADP/O ratio of rat liver mitochondria are shown in Table 1. Glyphosate up to 5 mM does not significantly affect the RCR and ADP/O. However, even at 0.5 mM (concentrations are expressed in terms of the final glyphosate concentration present in each assay) Roundup significantly depresses RCR and ADP/O ratio.

Fig. 2 shows the effects of Roundup and glyphosate on the respiratory rates characteristic of state 4 (succinate alone), ADP-stimulated respiration (state 3), and FCCP-stimulated respiration (uncoupled respiration) of rat mitochondria. Glyphosate did not show any significant effect on the respiratory rates. State 3 and uncoupled respiration rates were significantly depressed by Roundup for concentrations up to 15 mM, whereas state 4 respiration was stimulated twice as much.

Control experiments show that in the absence or in the presence of any of the used xenobiotics oxygen consumption was completely inhibited by the addition of antimycin A (not shown), indicating that the oxygen consumption occurred exclusively due to the respiratory activity.
3.2. Xenobiotic effects on mitochondrial membrane potential

The effects of Roundup and glyphosate on the energization and phosphorylation capacities of mitochondria were investigated through following the transmembrane potential (Δψ) developed by mitochondria upon succinate oxidation (Fig. 3A). The presence of glyphosate up to 15 mM did not affect the Δψ; on the other hand, up to 10 mM Roundup almost collapsed Δψ promoted by succinate although the dissipation, is already very significant for a concentration of 1 mM. After succinate addition, mitochondria developed a transmembrane potential of about −210 mV. Addition of ADP to succinate energized mitochondrial suspension causes a depolarization of Δψ to about −190 mV, since ATP-synthase uses Δψ to phosphorylate ADP. After a short lag phase, during which ADP

### Table 1

Effects of Roundup and glyphosate on succinate-dependent respiratory indexes RCR and ADP/O of rat liver mitochondria

<table>
<thead>
<tr>
<th>(mM)</th>
<th>Roundup</th>
<th>Glyphosate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ADP/O</td>
<td>RCR</td>
</tr>
<tr>
<td>0.00</td>
<td>1.81 ± 0.03</td>
<td>4.02 ± 0.04</td>
</tr>
<tr>
<td>0.50</td>
<td>1.56 ± 0.04*</td>
<td>3.06 ± 0.08*</td>
</tr>
<tr>
<td>1.00</td>
<td>1.53 ± 0.02*</td>
<td>3.14 ± 0.06*</td>
</tr>
<tr>
<td>2.00</td>
<td>1.48 ± 0.03*</td>
<td>3.00 ± 0.06*</td>
</tr>
<tr>
<td>5.00</td>
<td>1.13 ± 0.02*</td>
<td>2.11 ± 0.09*</td>
</tr>
</tbody>
</table>

The results correspond to the mean ± SD of four to six independent experiments.

* p < 0.05 compared with the control (in the absence of xenobiotics).
phosphorylation takes place, the transmembrane potential repolarizes back to the initial value. The presence of Roundup in the reaction medium depresses $\Delta \psi$ promoted succinate. Furthermore, Roundup not only decreases the depolarization and repolarization amplitude induced by ADP, but also lengthens the lag phase preceding repolarization (Fig. 3B), pointing to an inhibitory effect on phosphorylation efficiency which is also corroborated by the results obtained for ADP/O (Table 1).

### 3.3. Effects of the xenobiotics on the mitochondrial enzymatic activities

Studies of enzymatic activities of respiratory complexes II, III and IV localized the components of the mitochondrial respiratory chain affected by Roundup and glyphosate. Glyphosate, in the concentration up to 15 mM, does not affect any of the enzymatic activities of respiratory complexes (Fig. 4). Fig. 4 shows that the terminal segment of the chain, cytochrome $c$ oxidase (complex IV), was not affected by Roundup; however, succinate dehydrogenase (complex II) and succinate cytochrome $c$ reductase (complex III) were partially inhibited, indicating that Roundup interacts with electron transfer at the level of complex II and III.

Fig. 5 shows the effects of glyphosate and Roundup on the ATPase and ATP synthase activities. Glyphosate, in the concentration up to 15 mM, did not show any significant effect on the ATPase and ATP synthase activities. For concentrations up to 15 mM Roundup significantly depresses ATPase activity (60%), whereas up to 5 mM ATP synthase was almost inhibited (91%).

### 3.4. Mitochondrial swelling

In order to evidence the possible protonophoric properties of the tested xenobiotics, mitochondrial swelling experiments were performed in iso-osmolar medium in the presence of Roundup or glyphosate. The K$^+$ ionophore valinomycin was added in order to allow the movement of K$^+$ across the mitochondrial membrane. As illustrated in Fig. 6, under these experimental conditions, both Roundup (A) and glyphosate (B) do not induce a significant swelling. In fact, only for 15 mM of Roundup was the permeability of mitochondria protons slightly increased, a small effect compared with that of FCCP.

Fig. 7 shows that unlike glyphosate, Roundup, induces non-specific mitochondrial membrane permeabilization. The Ca$^{2+}$ concentration that should be applied to assess the effect of Roundup on PTP induction was evaluated and a Ca$^{2+}$ concentration of 50 $\mu$M was selected. In the presence of Roundup, Ca$^{2+}$-loaded mitochondria treated with RuRed underwent a permeability transition, as reflected by the decrease in absorbency at 520 nm, an effect that was completely inhibited by prior addition of Cyclosporine-A (CsA) (Zoratti and Szabo, 1995). Treatment of mitochondria with glyphosate concentrations up to 15 mM did not induce any swelling.
Discussion

The present study addresses the in vitro effect of Roundup and glyphosate on rat liver mitochondrial bioenergetics. Liver mitochondria preparation is a very convenient method for studying bioenergetic toxicities of xenobiotics (Da Silva et al., 1998; Peixoto et al., 2003); moreover, data from mitochondrial studies can generally be correlated with cytotoxicity parameters evaluated by other methods (Blondin et al., 1987; Knobeloch et al., 1990a,b).

Data obtained in this study clearly demonstrate the ability of Roundup to impair mitochondrial bioenergetic reactions. Alteration of basic mitochondrial functions was monitored by the detection of changes induced in mitochondrial respiration and membrane energization (Δψ). From the chemical structure of glyphosate, a protonophore action promoted by the phosphonic group cannot be taken into consideration since pK\textsubscript{a1} (2.0) and pK\textsubscript{a2} (2.25) values are too low (Wauchope, 1978). Nevertheless, the carboxylic group, which has a pK\textsubscript{a} (5.5) (Wauchope, 1978) value near that of acetic acid (acetic acid at pH 7.1 can transport protons across the membrane), could not participate in the transmembrane transport of protons, since at pH 7.1 the molecule of glyphosate should be almost charged ([H\textsubscript{3}A]	extsuperscript{-} 7.9 \times 10^{-6} [H\textsubscript{2}A\textsuperscript{-}]). As a result, glyphosate is not capable of crossing the lipidic membrane. Consequently, is not surprising that under these experimental conditions glyphosate does not have any significant effect on the mitochondrial state 4, on state 3, and on uncoupled respiration, whereas Roundup inhibited the state 3 and the uncoupled respiration. State 4 stimulation by Roundup was not higher since two antagonistic effects are observed; a mitochondrial membrane permeability which increases the respiratory rate and an inhibition of the succinate oxidation, which decreases the respiratory rate. From Fig. 2 (state 4 respiration in the presence of Roundup) we can see that the inhibitory effect is preponderant at higher concentrations.

A similar result was reported for potato mitochondria, where a pre-incubation period in the presence of glyphosate 0–50 mM did not reveal any measurable effect (Arnaud et al., 1993). However, with rat liver mitochondria glyphosate at a concentration of 50 mM reveals a small but significant inhibition on the mitochondrial bioenergetics (data not shown).
The in vitro effect of glyphosate on rat liver mitochondria is not completely concurrent with the results obtained in vivo (Olorunsogo et al., 1979). For in vitro assays, higher concentrations or a longer incubation period should probably be used in order to obtain a better correlation between in vivo and in vitro results. However, the assays with viable mitochondria cannot be longer than a few hours, which means that only acute effects can be studied.

Fig. 3A shows that Roundup promotes a strong decrease in mitochondrial transmembrane (Δψ) potential in a dose-dependent manner, with a maximal effect for 10 mM. Meanwhile glyphosate, even at 15 mM, does not affect the Δψ, which is in agreement with the results obtained for oxygen consumption. The observed decrease in the Δψ induced by Roundup cannot be attributed to a protonophoric action, since the valinomycin-induced swelling in KCl medium was not significantly induced by Roundup or glyphosate. Under these experimental conditions, the obtained results are not concurrent with the reported for Cyprinus carpio which were exposed by emersion in Roundup (Szarek et al., 2000). This should not come as a surprise though reported comparisons between species showed that the toxicity of Roundup or glyphosate varied according to species and developmental stage (Howe et al., 2004). The Δψ collapse promoted by Roundup could not be explained by a protonophoric action but it was certainly the result of a non-specific membrane permeabilization, referred to as mitochondrial permeability transition (MPT) (Bernardi, 1992) specifically inhibited by CsA (Zoratti and Szabó, 1995) and due to the inhibition of the complex II and III.

Roundup depresses the efficiency of the electron transport chain when succinate is the oxidative substrate, but does not affect the terminal segment of the chain. However, succinate dehydrogenase and succinate cytochrome c reductase are significantly inhibited by Roundup, suggesting that this herbicide affects the redox electron transfer chain at the level of complexes II and III (Fig. 4), although an effect on the translocation of succinate across the inner membrane cannot be overlooked. Glyphosate does not affect any of the studied respiratory complexes (Fig. 4).

The results obtained for ATP synthase (ATPase) system (Fig. 5) indicate that Roundup, though not glyphosate, at concentrations up to 15 mM, can directly interact with the enzymatic complex (ATPase/ATP synthase). However, from the difference observed between the inhibition promoted on the ATPase activity and in ATP synthase, it can be concluded that a significant part of the inhibition observed on the ATP synthase is merely the result of the inhibitory effect on the respiratory complexes II and III and due to non-specific inner mitochondrial membrane permeabilization.

Glyphosate presents a low partition coefficient in the mitochondrial membrane; considering that the greater part of the phosphorilative reactions are performed in the membrane. Any effect is the result of a much less xenobiotic concentration than that used in the assay. In fact, in potato tuber mitochondria incubated for 15 min in a medium containing 50 mM glyphosate, the membrane concentration may reach a maximum of 5 μM (Arnaud et al., 1993). It is, therefore, easy to understand why biochemical processes taking place inside the membrane (vectorial transport of protons, electron transfer) are not significantly affected by glyphosate.

The observed alterations in mitochondrial bioenergetics caused by Roundup cannot be exclusively attributed to the active ingredient, but may as well be the result of other chemicals e.g. POEA, or due the possible synergy between glyphosate and Roundup formulation products. This synergy between glyphosate and Roundup inert ingredients has also been reported in cell division (Marc et al., 2002), where the delay observed in the cell cycle could be the result of alterations on the mitochondrial bioenergetic reactivity, which haves drastic consequences on cellular function through the perturbation of the bioenergetic charge and balance of the cell. Therefore, the reduced energetic efficiency of mitochondria may account for some toxic effects resulting from the impairment of the energy requirements of the cell and from the crucial importance of energy metabolism in active tissues, e.g. liver.

In short, in this study, we found that the so called “inert ingredients” used in Roundup are greatly responsible for the observed toxicity at the bioenergetic level.

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