Vitamin C, resveratrol and lipoic acid actions on isolated rat liver mitochondria: all antioxidants but different

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Modulating mitochondrial antioxidant status is a nutritional issue of great interest in the treatment or prevention of several oxidative stress related diseases such as obesity. Thus, the aim of the present study was to analyze the effects of three antioxidants on hepatic mitochondrial function and antioxidant status. Isolated rat liver mitochondria were incubated with vitamin C, resveratrol and lipoic acid. The activity of antioxidant enzymes (manganese superoxide dismutase and glutathione peroxidase), ROS generation and respiratory parameters (RCR, P/O ratio and respiratory states) were measured. Vitamin C influenced mitochondrial function by decreasing of ROS generation ($P < 0.0001$), by stimulating the activity of manganese superoxide dismutase ($197.60 \pm 35.99\%$; $P < 0.001$) as well as glutathione peroxidase ($15.70 \pm 5.76\%$; $P < 0.05$) and by altering the activity of the electron transport chain, mainly by decreasing the P/O ratio ($P < 0.05$). Resveratrol induced a significant increase in manganese superoxide dismutase activity ($160 \pm 11.78\%$; $P < 0.0001$) and a decrease in ROS generation ($P < 0.05$ to $P < 0.0001$). By contrast, lipoic acid inhibited glutathione peroxidase activity ($16.48 \pm 3.27\%$; $P < 0.05$) and induced the uncoupling of the electron transport chain ($P < 0.01$). Moreover, this antioxidant induced a strong decrease in the P/O ratio ($P < 0.05$ to $P < 0.0001$). In conclusion, our results suggest that the three tested antioxidants produced direct effects on mitochondrial function, although the magnitude and intensity of these actions were significantly different, which may have implications when administrated as antioxidants.

Keywords: antioxidant, mitochondria, oxidative stress, reactive oxygen species

Introduction

Oxidative stress has been generating much recent

interest mainly because of its accepted role as a major contributor to the aetiology of several pathologies with serious public health implications such as

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Abbreviations: DHLA, dihydrolipoic acid; DIO, diet-induced obesity; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; IRLM, isolated rat liver mitochondria; LA, lipoic acid; MnSOD, manganese superoxide dismutase; $O_2^{\cdot-}$, superoxide anion; RCR, respiratory control ratio; ROS, reactive oxygen species; RSV, resveratrol; VC, vitamin C.

obesity,¹ diabetes² or metabolic syndrome.³ Oxidative stress can result from imbalance between reactive species production and antioxidant defenses.⁴ The mitochondrion is a major cellular site involved in energy metabolism, and it is also the main source of reactive oxygen species (ROS). In order to counter-balance the negative effects of ROS, mitochondria also contain enzymatic antioxidant defences, such as manganese superoxide dismutase (MnSOD) and glutathione peroxidase (GPx).⁵

The attenuation or complete suppression of oxidative stress as a way to improve several diseases has flourished as one of the main challenges of research in the last few years. Several approaches have been carried out either to decrease the high levels of ROS generated or boost the endogenous levels of antioxidants. Thus, numerous trials have examined the effects of supplementation with different antioxidants on oxidative stress.^{6,7} In this context, multiple lines of evidence suggest that vitamin C (VC), a hydrophilic vitamin, resveratrol (RSV), a natural polyphenol, and α -lipoic acid (LA), a short chain fatty acid, have beneficial effects on several pathologies related to oxidative stress.

In particular, a recent study showed a reduction in body weight gain and fat depots after supplementation with vitamin C.⁸ On the other hand, Baur *et al.*⁹ described the beneficial effects of RSV on life-span in obese mice. Finally, Prieto-Hontoria *et al.*¹⁰ demonstrated significant anti-obesity effects of LA in a diet-induced obesity rat model (DIO).

More specifically, these antioxidants showed direct effects on mitochondria. A study by Lowes *et al.*¹¹ demonstrated the key role of vitamin C on mitochondrial function. The beneficial effects of RSV on mitochondrial function have been thoroughly discussed in different research articles^{12–14} and LA is also known as an essential co-factor for mitochondrial bioenergetics enzymes¹⁵ corroborating its role in mitochondria.

Thus, this research attempted to address, apparently for the first time, the *in vitro* effects of vitamin C, RSV and LA on mitochondrial function by measuring the electron transport status, the efficiency of oxidative phosphorylation, ROS production and the activity of the main mitochondrial antioxidant enzymes.

Materials and methods

Animals

Thirteen male Wistar rats (12 weeks old) weighing 270–290 g (281.6 ± 3.76 g) were supplied by the Applied Pharmacobiology Center (CIFA, Pamplona, Spain).

The animals were housed in cages in a temperature controlled room ($22 \pm 2^\circ\text{C}$) with a 12:12 h light:dark cycle. Food and water were given *ad libitum*. All procedures were performed according to national and institutional guidelines of the Animal Care and Use Committee at the University of Navarra. Different animals were used to provide liver mitochondria for the measurement of respiratory parameters ($n = 6$) than those used for measurements of ROS production and enzymatic activities ($n = 7$).

Materials

ADP, succinate, rotenone, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), lucigenin, luminol, ascorbic acid, RSV and α -LA were obtained from Sigma-Aldrich Chemical Co (St Louis, MO, USA).

The concentrations used for the different antioxidants were calculated based on previous *in vitro* studies.^{13,14,16–20} Thus, 30 μM for vitamin C and lipoic acid and 50 μM for resveratrol were used. In particular, the concentration of RSV is slightly higher than the other two antioxidants since previous studies developed in primary rat hepatocytes have shown that the main effects of RSV were observed when using concentrations equal to or higher than 50 μM .²¹ RSV and LA were diluted in ethanol and an equal concentration of ethanol was added to the control. On the other hand, vitamin C was diluted in water and an equal concentration of water was also added to its corresponding control.

Mitochondrial preparations

Mitochondria were isolated by differential centrifugation as previously described Rickwood *et al.*,²² with minor modifications.²³ After sacrificing the animals by decapitation, livers were carefully minced with scissors and rinsed thoroughly in an isolation buffer (10 ml/g tissue) containing 250 mM sucrose, 1 mM EDTA and 5 mM NaTES, pH 7.4. The tissue was further homogenized in a Potter–Elvehjem homogenizer. The homogenate was centrifuged for 10 min at 3000 *g*. The pellet was discarded and the supernatant was recollected and centrifuged for 10 min at 14,500 *g*. The mitochondrial pellet was resuspended in buffer 2 which contained 250 mM sucrose, 5 mg/ml BSA-free fatty acids and 5 mM NaTES, pH 7.4. The homogenate was again centrifuged for 10 min at 3000 *g* and the supernatant was also centrifuged for 10 min at 14,500 *g*. The mitochondrial pellet was carefully resuspended in a minimal volume (0.5 ml) of buffer 2 and mitochondrial protein content was assayed by pyrogallol red method. All steps of mitochondrial isolation were performed at 4°C .

Incubation of isolated mitochondria with antioxidants

Rat liver mitochondria (IRLM) isolated by the procedure described before, were incubated with incubation medium (70 mM sucrose, 250 mM manitol, 2.5 mM KH_2PO_4 , 2.5 mM MgCl_2 , 0.5 mM EDTA, 2 mM HEPES and BSA 0.1%, pH 7), in the absence (control group) or in the presence of the antioxidants; vitamin C, RSV and LA.

The incubation periods were determined after testing different times (0 to 360 min) and 30 min and 60 min were selected. The maximum incubation time was established at 60 min because at higher incubation periods the electron transport chain was uncoupled. All measurements were performed in triplicate.

Chemiluminescent measurement of superoxide and hydrogen peroxide production by IRLM

Superoxide production was quantified by measuring the lucigenin-derived chemiluminescence by a Luminoskan Ascent (Thermo Electron Corporation) at room temperature. The reaction mixture contained 0.5 mg of mitochondrial protein, 250 μl of chemiluminescence buffer and an exogenous substrate (5 mM succinate). The lucigenin-derived chemiluminescence was initiated by adding a final concentration of 5 μM lucigenin and continuously monitored for up to 60 min. Several investigations have described that a lucigenin concentration lower than 50 μM did not stimulate redox cycling;^{24,25} thus a concentration of 5 μM (10-fold lower) has been used in this study.²⁴

For hydrogen peroxide production measurement, the luminol-derived chemiluminescence was quantified by a Luminoskan Ascent (Thermo Electron Corporation) at room temperature. Mitochondrial protein (0.1 mg) was mixed with a buffer, succinate (5 mM) and horseradish peroxidase (10 $\mu\text{g}/\text{ml}$). The reaction of chemiluminescence was initiated by adding of 5 μM luminol and was continuously monitored for 60 min.

In order to discriminate a possible error caused by the potential reactivity of the buffers with lucigenin and luminol, we determined a background by measuring the chemiluminescence produced in mixtures with no mitochondria. The values obtained were subtracted from the values obtained when mitochondria were added to these mixtures.

Activity of MnSOD and GPx in IRLM

MnSOD activity in IRLM was measured with a colorimetric activity kit (Superoxide Dismutase Activity Kit. Cat. n° 900-157, Assay Designs Inc., MI, USA). This colorimetric assay was based on the following reactions: first, $\text{O}_2^{\bullet-}$ was generated from the

conversion of xanthine and oxygen to uric acid and hydrogen peroxide catalyzed by xanthine oxidase. The $\text{O}_2^{\bullet-}$ then converts WST-1 to WST-1-formazan, a coloured product that absorbs light at 450 nm. Finally, SOD reduces the $\text{O}_2^{\bullet-}$ concentration and thereby lowers the rate of WST-1-formazan formation. Reduction in the appearance of WST-1-formazan is a measure of SOD activity present in the mitochondria.²⁶ In other words, relative SOD activity of the experimental sample is determined from percentage inhibition of the rate of formation of WST-1-formazan.

GPx activity in IRLM was measured using a colorimetric activity kit assay (Glutathione Peroxidase Activity Kit. Cat. n° 900-158, Assay Designs Inc.). This method is based on the reaction of reduced glutathione (GSH) with H_2O_2 , which produces oxidized glutathione (GSSG). This reaction is catalyzed by GPx. GSH is regenerated by the reaction of GSSG with NADPH which, in turn, leads to the production of NADP^+ . The oxidation of NADPH to NADP^+ is accompanied by a decrease in absorbance at 340 nm (A_{340}). The rate of decrease in the A_{340} is directly proportional to the glutathione peroxidase activity in the mitochondria.

Oxygen consumption

Mitochondrial oxygen consumption was polarographically measured with a Hansatech (UK) Clark-type oxygen electrode (Respire1), whose incubation chamber was thermostatically set at 30°C and magnetically stirred. Fresh IRLM (1 mg/ml) were incubated in a respiration medium containing 75 mM NaCl, 1 mM EGTA, 10 mM NaTES, 2 mM NaH_2PO_4 and 1 mg/ml BSA FFA.²³ Succinate (2.5 mM) was added in order to mimic the formation of intermediates of the citric acid cycle as occurs *in vivo*. Addition of ADP (0.1 mM) initiates state 3 respiration whereas state 4 is established when ADP is phosphorylated and converted into ATP. Finally, maximal oxygen flux (state uncoupled) was obtained by titration with the chemical uncoupler FCCP.

The mitochondrial function and coupling of the electron transport chain was evaluated by the respiratory control ratio (RCR) calculated according to Estabrook.²⁷ The P/O ratio was also determined. This ratio has been defined as the amount of oxygen consumed during respiratory state 3, which is the amount of oxygen used to drive ATP synthesis and proton leakage across the mitochondrial membrane. In this context, the P/O ratio represents an indirect measure of the efficiency of mitochondrial oxidative phosphorylation.^{28,29}

Statistical analyses

Data are reported as mean $\pm$ SE. Normal distribution was confirmed by two different methods, Shapiro–Wilk and Kolmogorov–Smirnov. In order to determine the effects of different treatments, repeated measures ANOVA followed by post-hoc comparisons by Dunnet's test were carried out. The interactions between incubation periods and treatments were analyzed by two-way ANOVA. Relationships between variables were analyzed by calculating Pearson correlation coefficients. All statistical analyses were performed using GraphPad Prism v5.0 software.

Differences were considered as statistically significant at $P < 0.05$.

Results

Effects of vitamin C, resveratrol and lipoic acid on ROS production in IRLM

All the antioxidants tested (vitamin C, RSV and LA) significantly decreased superoxide ($O_2^{\bullet-}$) production as observed in Figure 1 ($P < 0.05$ to $P < 0.0001$) after both 30 min and 60 min of incubation. However, the magnitude of these inhibitory effects was different.

Figure 1 Effects of different antioxidants on mitochondrial superoxide production in isolated rat liver mitochondria after 30 min and 60 min of incubation. Values shown are mean $\pm$ SE of integrated areas under curve of lucigenin-derived chemiluminescence per mg of protein ($n = 7$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$ vs control group at each time according to repeated measure ANOVA with Dunnet's post-hoc analysis

Figure 2 Effects of different antioxidants on mitochondrial hydrogen peroxide production in isolated rat liver mitochondria after 30 min and 60 min of incubation. Values shown are mean $\pm$ SE of integrated areas under curve of lucigenin-derived chemiluminescence per mg of protein ($n = 7$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$ vs control group at each time according to repeated measure ANOVA with Dunnet's post-hoc analysis

Thus, the decrease induced by vitamin C was more potent than those observed after treatment with RSV or LA.

Interestingly, hydrogen peroxide (H_2O_2) production was affected in a different way depending on the antioxidant analyzed (Fig. 2). Thus, our data showed that vitamin C and RSV induced a significant inhibition ($P < 0.05$ to $P < 0.0001$) in the production of this reactive species at both incubation periods. However, treatment with LA showed a tendency to increase H_2O_2 generation after both incubation periods, although this increase did not reach statistical significance.

Effects of vitamin C, resveratrol and lipoic acid on mitochondrial antioxidant enzymes

Vitamin C strongly stimulated MnSOD activity after all periods of incubation tested, being the highest stimulatory effect after 30 min of treatment ($197.60 \pm 35.99\%$; $P < 0.0001$). RSV significantly increased the activity of MnSOD after 30 min and 60 min of treatment ($160 \pm 11.78\%$, $P < 0.0001$ and $47.15 \pm 15.33\%$, $P < 0.01$, respectively) and a significant interaction between incubation time and treatment was observed ($P < 0.0001$). In contrast, LA treatment did not affect the activity of this antioxidant enzyme (Fig. 3A).

Regarding the effects of these antioxidant on GPx activity, vitamin C treatment induced an increase after 30 min ($26.18 \pm 17.72\%$) and 60 min of treatment ($15.70 \pm 5.76\%$) but statistical differences were only observed after 60 min of incubation ($P < 0.05$). Moreover a positive correlation between H_2O_2 production and the activity of this enzyme was observed ($P = 0.0008$; $r = 0.8313$). On the other hand, the effects of RSV on GPx activity were affected by the incubation time. In fact, RSV induced a significant decrease in GPx activity after 30 min of incubation ($19.80 \pm 7.60\%$; $P < 0.01$), although these effects disappeared after 60 min. Finally, treatment with LA has an inhibitory effect on the activity of this enzyme after short incubation periods in comparison to the control ($16.48 \pm 3.27\%$; $P < 0.05$; Fig. 3B).

Effects of vitamin C, resveratrol and lipoic acid on respiratory parameters

The direct effects of these antioxidants on different parameters of the mitochondrial electron transport chain and oxidative phosphorylation were also investigated. Thus, treatment with vitamin C showed a tendency to induce an increase in the RCR after 60 min (Table 1). This increase seems to be a consequence, at least in part, of the significant

Figure 3 Influence of vitamin C (VC), RSV and LA on the activity of the main mitochondrial antioxidant enzymes: MnSOD (A) and GPx (B) in isolated rat liver mitochondria ($n = 7$) after 30 min and 60 min of incubation. Values shown are mean $\pm$ SE of integrated areas under curve of lucigenin-derived chemiluminescence per mg of protein ($n = 7$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$ vs control group at each time according to repeated measure ANOVA with Dunnet's post-hoc analysis

reduction observed in oxygen consumption on respiratory state 4 ($P < 0.05$; Table 1). In addition, the P/O ratio was significantly reduced by vitamin C after 30 min and 60 min of incubation ($P < 0.05$; Fig. 4A). A significant reduction in respiratory state 4 after 30 min of incubation was also observed after RSV treatment ($P < 0.05$) as well as a significant decrease in the P/O ratio, but only at longer incubation periods ($P < 0.05$; Fig. 4B). Finally, treatment of IRLM with LA

Table 1 Changes in respiratory parameters after incubation of IRLM with vitamin C, RSV and LA

Incubation time	Control	Vitamin C (30 μ M)	RSV (50 μ M)	LA (30 μ M)	ANOVA
30 min					
RCR	3.63 $\pm$ 0.35	3.29 $\pm$ 0.71 (ns)	3.26 $\pm$ 0.64 (ns)	2.71 $\pm$ 0.76**	0.0048
State 2 (nmolO ₂ /min/mg)	28.17 $\pm$ 4.40	23.59 $\pm$ 9.22 (ns)	23.03 $\pm$ 8.87 (ns)	29.78 $\pm$ 10.4 (ns)	0.0323
State 3 (nmolO ₂ /min/mg)	81.34 $\pm$ 7.97	75.06 $\pm$ 18.7 (ns)	73.73 $\pm$ 9.79 (ns)	72.66 $\pm$ 9.50 (ns)	0.2787
State 4 (nmolO ₂ /min/mg)	31.37 $\pm$ 6.24	25.02 $\pm$ 10.6*	23.88 $\pm$ 6.55*	28.89 $\pm$ 11.1 (ns)	0.0117
60 min					
RCR	3.11 $\pm$ 0.39	3.41 $\pm$ 0.33 (ns)	3.16 $\pm$ 0.38 (ns)	2.16 $\pm$ 0.64**	0.0003
State 2 (nmolO ₂ /min/mg)	19.93 $\pm$ 8.20	20.12 $\pm$ 3.37 (ns)	21.51 $\pm$ 4.12 (ns)	20.48 $\pm$ 4.46 (ns)	0.9354
State 3 (nmolO ₂ /min/mg)	71.23 $\pm$ 7.92	70.63 $\pm$ 10.2 (ns)	69.76 $\pm$ 8.30 (ns)	55.31 $\pm$ 4.57**	0.0071
State 4 (nmolO ₂ /min/mg)	27.36 $\pm$ 3.85	23.32 $\pm$ 3.98*	24.08 $\pm$ 4.38 (ns)	23.74 $\pm$ 3.88 (ns)	0.0501

Values shown are mean $\pm$ SE ($n = 6$). ns, not significant; * $P < 0.05$; ** $P < 0.01$ vs control group at each time, according to repeated measure ANOVA with Dunnet's post-hoc analysis. Respiratory state 2 was established in presence of oxidizable substrates (succinate 2.5 mM plus rotenone 5 μ g/ μ l). Respiratory state 3 was measured in presence of ADP 0.1 mM. Respiratory state 4 was established after ADP was phosphorylated to ATP.

induced the strongest effects on respiratory parameters in comparison with the other two antioxidants analyzed. Thus, LA induced a significant decreased in RCR after 30 min and 60 min ($P < 0.01$), which seems to be secondary to the decrease observed on oxygen consumption in respiratory state 3 (Table 1). Furthermore, a significant reduction in the P/O

ratio was also found after treatment with this antioxidant ($P < 0.05$ to $P < 0.0001$; Fig. 4C).

Discussion

Antioxidants are defined as 'any substance that when present at low concentrations compared with those of

Figure 4 Changes in P/O ratio after treatment with vitamin C (A), resveratrol (B) and lipoic acid (LA) at different incubation periods ($n = 6$). Values shown are mean $\pm$ SE of integrated areas under curve of lucigenin-derived chemiluminescence per mg of protein ($n = 7$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$ vs control group at each time according to repeated measure ANOVA with Dunnet's post-hoc analysis

an oxidizable substrate significantly delays or prevents oxidation of that substrate'.³⁰ However, herein, we have demonstrated for the first time that not all antioxidants behave in the same way or with the same intensity, at least when their direct actions on mitochondria are analyzed.

Vitamin C is one of the most widely used dietary supplements due to its antioxidant properties. The ability of vitamin C to reduce and neutralize ROS has been widely described.³¹ Our data showed that treatment of IRLM with vitamin C (30 μ M) induced a significant decrease in the levels of $O_2^{\cdot-}$, which was corroborated by Oliveira *et al.*³² These beneficial effects could be explained by the stimulation exerted by this vitamin on MnSOD activity, suggesting that $O_2^{\cdot-}$ is rapidly converted into H_2O_2 . In this sense, previous studies have shown that the protective role of vitamin C against oxidative stress is mediated by the stimulation of major mitochondrial antioxidant enzymes.^{11,33}

The beneficial effects of vitamin C on oxidative damage can also be justified by its capacity to modulate H_2O_2 production, as demonstrated in several studies³⁴ as in ours. H_2O_2 generation has been associated with oxygen consumption in respiratory state 4.³⁵ In this sense, we observed a significant reduction in H_2O_2 production and in oxygen consumption in state 4, suggesting that the decrease detected in H_2O_2 after treatment with vitamin C may be secondary to the reduction in oxygen consumption in this respiratory state. Another mechanism that could explain the reduction found in H_2O_2 generation after vitamin C treatment is the increased activity of GPx, which catalyze the detoxification of H_2O_2 to H_2O .

We have also investigated the effects of vitamin C on the functionality and efficiency of the electron transport chain. Our data showed an increase in the RCR after 60 min of treatment with vitamin C, which could be due, at least in part, to the reduction observed in oxygen consumption in state 4. Moreover, vitamin C induced a significant decrease in the P/O ratio, which has been established as a marker of oxidative phosphorylation efficiency.²⁸ These results might suggest that vitamin C may have a potential positive role in the treatment of obesity by decreasing metabolic efficiency. Indeed, recent investigations showed a decrease in metabolic efficiency in an animal model of DIO supplemented with this vitamin.³⁶

These data show that vitamin C exerts these positive effects on mitochondrial redox state by

inducing the activity of the electron transport chain, which is supported by the increase observed in the RCR, as found by Mandl *et al.*³⁷ This increase in the electron transport chain activity gives support to findings concerning to the decrease in $O_2^{\cdot-}$ levels observed in isolated mitochondria treated with vitamin C. Furthermore, this increase in electron transport chain activity provides electrons to ascorbic acid in order to be transformed into dihydroascorbic acid, as previously proposed by Mandl *et al.*³⁷ In addition, several studies have reported that vitamin C is a natural scavenger of ROS,³⁸ being able to increase MnSOD and GPx expression and activity,^{33,39} which, in turn, leads to a decrease in superoxide and hydrogen peroxide production, respectively.

RSV has been reported to improve mitochondrial function.^{12,40} The capacity of this antioxidant to reduce ROS levels has been proposed as a mechanism that could explain its protective role against situations with an imbalance in the oxidative status.⁴¹ In this sense, a reduction in $O_2^{\cdot-}$ generation was observed in our study. This reduction seems to be secondary to the increase observed in MnSOD activity, as previously reported with vitamin C. Furthermore, different studies have proposed that the beneficial effects of RSV against oxidative stress damage are mediated by a specific and progressive stimulation of MnSOD.⁴² Furthermore, RSV showed a very potent inhibitory action on H_2O_2 production. Our findings were corroborate by Brookins Danz *et al.*,¹² who observed a decrease in basal production of H_2O_2 after treatment with RSV in neonatal rat ventricular myocytes. This decrease could be explained by the reduction in respiratory state 4, in fact, and as with vitamin C, a significant decrease in H_2O_2 generation and in state 4 were observed. Conversely, changes in H_2O_2 production could not be explained by variations in GPX activity in our study. In fact, our results showed a decrease in the activity of this enzyme after 30 min of exposure to the antioxidant; however, this effect disappeared with longer incubation periods. Previous studies showed conflicting results regarding the action of RSV on the activity of GPx, suggesting that its effects depends on the tissue, dose and period of treatment.^{42,43}

Concerning the actions of RSV on the functionality and efficiency of the electron transport chain, this polyphenol did not show any effects on RCR, although a significant decrease in a P/O ratio was found. Thus, our results suggest that main effects of RSV in IRLM are the direct and progressive activation of MnSOD which is supported by data

from Robb *et al.*^{13,42} In addition, several investigations have proven the potent effect of RSV to scavenge ROS,⁴³ which corroborates the decreased production of both superoxide and hydrogen peroxide species observed in our study.

LA has been defined as the 'antioxidant of antioxidants',⁴⁴ because of its ability to decrease ROS production⁴⁵ and to stimulate the activity of antioxidant enzymes.⁴⁶ Thus, investigations carried out in mitochondria isolated from rat cardiomyocytes found a decline in the levels of $O_2^{\cdot-}$ after treatment with different doses of DHLA, which is the reduced form of LA.⁴⁷ Accordingly, our data showed a decrease in the levels of $O_2^{\cdot-}$ after treatment with LA, although this decrease can not be explained by variations in MnSOD activity. On the other hand, these results could be explained by different mechanisms associated with redox reactions that maintain the balance between the LA and DHLA.⁴⁸ In fact, some research described the capacity of DHLA to neutralized $O_2^{\cdot-}$; therefore, our investigation could suggest that the decrease observed in $O_2^{\cdot-}$ could be due, at least in part, to the transformation of LA into DHLA.

Despite these findings, it has also been established that LA may have pro-oxidant effects depending on its concentration, incubation time and the experimental model used.⁴⁹ In fact, our results showed a stimulatory action of LA on H_2O_2 generation, at all incubation periods tested, similar to the data provided by Dicter *et al.*⁵⁰ The increased levels of H_2O_2 could be explained by the reduction observed in the activity of GPx, which, in turn, could be due to a displacement of the GSH:GSSG balance to the oxidized form, as described by Morkunaite *et al.*⁵¹ However, we have not addressed this hypothesis.

Regarding the effects of LA on the electron transport chain, our data revealed a significant decrease in RCR, suggesting that lipoic acid could uncouple the electron transport chain. In fact, similar data were observed by Schönheit *et al.*⁴⁷ We consider that the uncoupling effect of lipoic acid on electron transport chain is the strongest effect of this molecule in IRLM. The data are consequently in agreement with other research that has described this effect of LA.⁵⁰ Our data also showed a decrease in the P/O ratio after treatment with LA corroborating previous studies.⁴⁷ These findings could suggest a potential therapeutic role of LA in the treatment of obesity. However, these results should be interpreted with caution since LA might have pro-oxidant and antioxidant properties in different circumstances.

Conclusions

Vitamin C improved mitochondrial function by decreasing ROS levels and by increasing the activity of antioxidant enzymes and the activity of the electron transport chain. The magnitude of the beneficial effects of RSV was lower than those observed for vitamin C, probably because other factors such as sirtuins could also be involved. By contrast, treatment with LA induced an apparently pro-oxidant effect on IRLM. The main mechanisms involved in this pro-oxidant effect could be summarized as the induction of an uncoupling of the electron transport chain and the stimulation observed in H_2O_2 generation, which seems to be due to the inhibition of GPx activity.

Finally, this study demonstrated that vitamin C, RSV and LA exerted direct effects on mitochondrial function, although the magnitude and intensity of these effects were significantly different. Furthermore, LA appears to exert some pro-oxidant actions, suggesting that not all antioxidants are equal or their effects are mediated by the same pathways. In fact, the outcomes after incubation of mitochondria with the three antioxidants should be carefully examined since the physical properties of the assayed molecules are different and could affect their ability to enter the mitochondria and, therefore, to affect their functionality. However, it is important to state that LA showed quite different actions in comparison with vitamin C and RSV even though both LA and RSV are lipophilic molecules, suggesting that other mechanisms different from pure physical characteristics of the compounds are involved in their effects on mitochondria. In fact, this is not the first time that compounds with similar chemical function demonstrate differential biological activities. Thus, Perez-Matute *et al.*⁵² demonstrated different actions on basal leptin production between linoleic acid and its isomer conjugated linoleic acid. Taking in to account these findings, more *in vivo* studies are needed in order to elucidate the beneficial effects of vitamin C, RSV and LA on mitochondrial function and oxidative stress associated diseases such as obesity.

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