Eur J Appl Physiol (2006) 98:263–269
DOI 10.1007/s00421-006-0273-y

ORIGINAL ARTICLE

Response of antioxidant defences to oxidative stress induced by prolonged exercise: antioxidant enzyme gene expression in lymphocytes

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Accepted: 6 July 2006 / Published online: 9 August 2006

Abstract The response of lymphocyte and plasma antioxidant defences to a prolonged exercise as a cycling stage in a professional race was analysed. Antioxidant enzyme activities and gene expression, carbonyl derivative and MDA levels were determined in lymphocytes. Plasma levels of vitamin E, carotenes, protein carbonyl derivatives and the test d-Roms were measured. Significant increases in plasmatic carbonyls and in the test d-Roms were observed after the cycling stage. No significant differences were found in the lymphocyte MDA and carbonyl derivative levels. A significant increase was found in plasma vitamin E concentration after the cycling stage; however, the lymphocyte vitamin E concentration did not change. Significant increases were observed in lymphocyte total superoxide dismutase (SOD) activity and in the levels of CuZn-SOD and Mn-SOD isoenzymes. The moderate levels of oxidative stress in the lymphocyte induced a cellular adaptation to exercise enough to counteract the negative effects of oxidative stress.

Keywords Cycling · Oxidative stress · Lymphocytes · Antioxidants

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Introduction

Modest amounts of regular exercise are generally associated with well being and a decreased risk of upper respiratory tract infections (Nieman 1994). However, strenuous exercise causes oxidative stress, resulting in lipid peroxidation, DNA damage and protein oxidation (Packer 1997). Specific sources of reactive oxygen and nitrogen species (ROS) during exercise include leakage of electrons from the mitochondrial electron transport chain, xanthine oxidase reaction, haemoglobin oxidation and activated neutrophils (Ji 1999; Sjodin et al. 1990). Exercise-induced ROS are also thought to modulate acute-phase inflammatory responses (Cannon and Blumberg 2000) and to have a role in cell signaling (Jackson 1999; Reid et al. 1992).

A heavy schedule of training and competition can lead to immune impairment in athletes, which is associated with an increased susceptibility to infections, particularly in the upper respiratory tract. This exercise-

induced immune dysfunction seems to be mostly due to the immunosuppressive actions of stress hormones such as adrenaline and cortisol (Gleeson et al. 2004).

The lymphocyte antioxidant defenses -which include large amounts of vitamin C and E but also enzymes such as catalase, superoxide dismutase (SOD) and glutathione peroxidase-, have shown adaptations to oxidative stress. Increases in vitamin E contents and in antioxidant enzyme activities after different bouts of strenuous exercise have been reported (Cases et al. 2005; Tauler et al. 2005). Furthermore, increases in lymphocyte heme oxygenase-1 (HO-1) mRNA expression after different exercises (Niess et al. 1999; Thompson et al. 2005), but also in HO-1 protein levels 2 h after exercise were found (Thompson et al. 2005). The mechanisms underlying these adaptations are unknown. However, it was recognised that free radical species can act as signals to modify directly or indirectly gene expression patterns (Jackson 1999; Reid et al. 1992).

Main changes in the protein and the mRNA levels of several antioxidants have been reported after exercise (Niess et al. 1999; Thompson et al. 2005). However, studies trying to correlate such changes to exercise intensity are still lacking. The aim of this study was to determine the response of plasma and lymphocyte antioxidant defenses to a prolonged exercise as a cycling stage in a professional race. The data obtained were compared with others obtained from similar subjects participating in a mountain cycling stage (Tauler et al. 2005), which is a more strenuous and demanding exercise.

Materials and methods

Subjects and exercise

Seven voluntary male subjects participated in this study. They were all professional cyclists participating in the "Challenge Volta a Mallorca 2003", a five-day competition for professional cyclists. Subjects were informed of the purpose of this study and the possible risks involved before getting their written consent to participate. The study protocol was in accordance with the Declaration of Helsinki and was approved by the local ethics committee (Ethical Committee of "University Hospital Son Dureta", Mallorca, Spain).

The study was developed during the second and the fourth stages of the "Challenge Volta Ciclista a Mallorca 2003". These cycling stages were similar (164.5 and 166.3 Km, respectively) and did not include mountainous terrain. The seven cyclists participated in two stages. The mean duration to complete these stages was 255 and 234 min, respectively.

Experimental procedure

Venous blood samples were taken from the antecubital vein with suitable vacutainers with EDTA as anticoagulant. Venous samples were taken following an overnight fast, before the race and 3 h after finishing the cycling stage.

Blood samples were used to purify lymphocytes and to obtain plasma. Blood cells were quantified in an automatic flow cytometer analyser Technicon H*2 (Bayer) VCS system. Antioxidant (catalase, glutathione peroxidase and SOD) enzyme activities, enzyme gene expression as well as carbonyl derivative and MDA levels were determined in lymphocytes. We determined vitamin E, carotenes and protein carbonyl derivative levels in plasma. The ROS and their derivative levels in serum were measured as the test d-Roms. Serum cortisol levels were also determined.

Lymphocyte and plasma purification

The lymphocyte fraction was purified following an adaptation of the method described by Boyum (1964). Blood was carefully introduced on Ficoll in a proportion of 1.5:1 and centrifuged at 900 *g*, at 4°C for 30 min. The lymphocyte layer was carefully removed. The plasma and the Ficoll phases were discarded. The lymphocyte slurry was then washed twice with PBS and centrifuged for 10 min at 1,000 *g*, 4°C. The cellular precipitate of lymphocytes was lysed with distilled water.

Plasma was obtained after centrifugation (15 min, 1,000 *g*, 4°C) of another blood sample obtained as above and was stored at −80°C until use.

Determination of serum lipid and cholesterol parameters

Serum triglycerides, total cholesterol, and HDL-cholesterol were determined by colorimetric methods using autoanalyzer DAX-72 (Technicon, Bayer). Triglycerides were hydrolyzed to glycerol and fatty acids using lipoprotein lipase. Glycerol, in a reaction catalyzed by glycerol kinase, is converted to glycerol-3-phosphate. In a third reaction, glycerol-3-phosphate is oxidized by glycerol phosphate oxidase to dihydroxyacetone phosphate and hydrogen peroxide. Finally, in a reaction catalyzed by peroxidase, hydrogen peroxide reacts with 4-aminoantipyrine and *N*-ethyl-*N*-sulfohydroxypropyl-*m*-toluidine sodium salt (TOOS) to form a chinoneimide whose absorbance can be measured at 524 nm (Tietz 1999). To obtain total cholesterol, serum samples were hydrolyzed with cholesterol esterase. Then, in the presence of oxygen, cholesterol

oxidase oxidizes free cholesterol to cholest-4-ene-3-one and hydrogen peroxide. The color reaction using peroxidase is the same as above. Cholesterol bound to high density plasma lipoproteins (HDL-cholesterol) was measured as the total cholesterol after immunoinhibition of LDL, VLDL and chylomicrons with anti-β-lipoproteins, as antibody to avoid the binding of lipoproteins to the enzymes used (Burtis and Ashwood 1984).

VLDL-cholesterol and LDL-cholesterol were calculated according to the Friedewald equations (Tietz 1999): [VLDL-cholesterol = triglycerides/5] and [LDL-cholesterol = total cholesterol − (HDL-cholesterol + VLDL-cholesterol)].

Determination of serum cortisol levels

Serum cortisol levels were determined by using an EIA assay in an autoanalyser (Bayer) (Weeks and Woodhead 1984).

Test d-Roms

The reactive oxygen metabolites (ROMs) were measured as marker of oxidative stress in serum by a colorimetric assay kit (Diacron, Grosseto, Italy). Because of the extreme ROS reactivity, they form derivatives in plasma that maintain a high chemical reactivity and good oxidising power. These chemical compounds, reacting with a specific buffered chromogen, give a colored complex. The concentration of this complex is proportional to the ROS derivatives. Results are expressed by using arbitrary units (U. Carr) (Cesarone et al. 1999).

MDA and protein carbonyl derivatives determination

MDA as marker of lipid peroxidation was analyzed in lymphocytes by a colorimetric assay kit (Calbiochem, San Diego, CA, USA). The method used is specific for MDA determination.

Protein carbonyl derivatives were measured both in plasma and in lymphocytes by an adaptation of the method of Levine et al. (1994). After deproteinizing the samples with trichloroacetic acid, the precipitates were resuspended with 2,4-dinitrophenylhydrazine (DNPH) 10 mM, and incubated for 60 min at 37°C. The samples were then, precipitated with 20% trichloroacetic acid, and centrifuged for 10 min at 1,000 *g* and 4°C. The precipitate was washed twice with ethanol:ethyl acetate (1:1) to remove free DNPH. Guanidine 6 M in phosphate buffer 2 mM, pH 2.3 was added to the precipitate, and samples were incubated for 40 min at 37°C. Finally, samples were centrifuged for 5 min at 3,000 *g* at 4°C to clarify the supernatant and the absorbance was measured at 360 nm.

Vitamin E and carotenes determination

Vitamin E was determined in plasma and lymphocytes. Carotenes were determined in plasma. The deep-frozen plasma or lymphocyte suspensions were thawed and mixed to disperse possible precipitates. The extraction of liposoluble vitamins and carotenoids was carried out using *n*-hexane after deproteinization with ethanol. Liposoluble vitamins and carotenoids were determined by HPLC in the *n*-hexane extract of plasma after drying under a nitrogen current and redissolving in methanol (Tauler et al. 2002). The mobile phase consisted of 550:370:80 acetonitrile:tetrahydrofuran:H_2O. The HPLC was a Shimadzu with a diode array detector and the column was a Nova Pak, C18, 3.9 × 150 mm. α-Tocopherol were determined at 290 nm. Cryptoxanthin, β-carotene and lycopene were determined at 460 nm; lutein/zeaxanthin was determined at 450 nm.

Lymphocyte antioxidant enzyme activities

Catalase, glutathione peroxidase, glutathione reductase and superoxide dismutase activities were determined in lymphocytes using a Shimadzu UV-2100 spectrophotometer at 37°C.

Catalase activity was measured by the spectrophotometric method of Aebi (Aebi 1984) based on the decomposition of hydrogen peroxide (H_2O_2). Glutathione reductase activity was measured following the Goldberg and Spooner (Goldberg and Spooner 1985) spectrophotometric method. Glutathione peroxidase activity was measured using the spectrophotometric method of Flohé and Gunzler (Flohe and Gunzler 1984). SOD activity was measured using the method of McCord and Fridovich (McCord and Fridovich 1969).

Lymphocyte RNA extraction and relative quantitative RT-PCR assay

Catalase, glutathione peroxidase and SOD mRNA expression was determined by multiplex real time RT-PCR using human 36B4 rRNA as reference. For this purpose, total RNA was isolated from lymphocytes by Tripure extraction (Roche Diagnostics, Germany). RNA (1 μg) from each sample was reverse transcribed using 50 U of Expand Reverse Transcriptase (Roche Diagnostics, Germany) and 20 pmol oligo (dT) for 60 min at 37°C in a 20 μl final volume, according to

manufacturer instructions. The resulting cDNA (0.5 µl) was amplified using the LightCycler FastStart DNA Master^{PLUS} SYBR Green I kit (Roche Diagnostics, Germany). Amplification was performed at 55°C and 40 cycles. The relative quantification was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$. Antioxidant enzyme levels before and alter the stage were normalised to the invariant control 36B4 rRNA. mRNA levels at the beginning of the stage were arbitrarily referred to as 1. Primers used are listed in Table 1.

Statistical analysis

Statistical analysis was carried out using a statistical package for social sciences (SPSS 9 for windows). Results are expressed as means ± SEM and $P < 0.05$ was considered statistically significant. All the data were tested for their normal distribution. Student's *t*-test for paired data was used to determine the significance of the data.

Results

The results obtained in each stage were similar. This observation allowed us to analyse the results as an only exercise.

As indicators of the occurrence of oxidative damage in plasma during the cycling stage, changes in plasma oxidative stress markers are shown in Fig. 1. Significant increases in plasmatic carbonyls (13%) and in the test d-Roms (24%) were observed after the cycling stage.

The changes in the lymphocyte number, the serum cortisol levels as well as in the oxidative damage markers in lymphocytes are reported in Table 2. The cycling stage did not induce any changes in the lymphocyte number. However, the serum cortisol levels decreased significantly (76.7%) after the stage. No significant differences were found in the lymphocyte MDA and carbonyl derivative levels.

As it is shown in Table 3 no significant differences were found after the cycling stage in the serum cholesterol, triglycerides and lipoprotein-cholesterol levels.

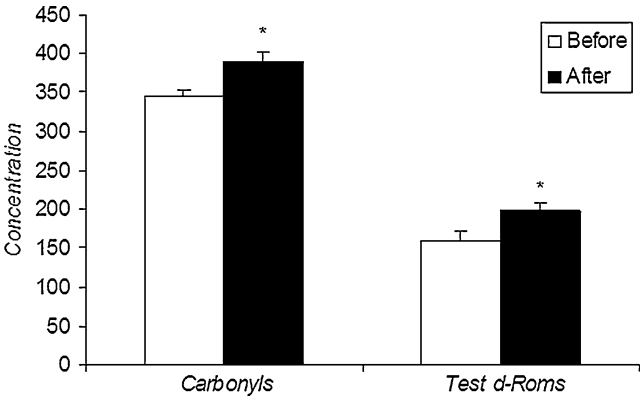

Fig. 1 Changes in plasma oxidative stress markers during the cycling stage. Indicates significant different values (Student's *t* test paired data, $P < 0.05$). Units: Carbonyls (µmol/l); Test d.Roms (U. Carr)

Table 2 Serum cortisol, lymphocyte number and lymphocyte oxidative damage markers before and after the cycling stage

	Before	After
Serum cortisol (µg/dl)	22.7 ± 0.8	5.29 ± 0.62*
Lymphocytes		
10^3/µl blood	2.72 ± 0.18	2.47 ± 0.14
MDA (µmol/10^9 cells)	0.23 ± 0.05	0.30 ± 0.04
Carbonyls (µmol/10^9 cells)	5.55 ± 0.33	6.02 ± 0.38

Significant different values (Student's *t* test paired data, $P < 0.05$)

Table 3 Serum levels of triglycerides, total cholesterol and lipoprotein-cholesterol during cycling stage

	Before	After
Cholesterol (mg/dl)	164 ± 4	166 ± 4
Triglycerides (mg/dl)	82.7 ± 16.6	106 ± 17
VLDL-cholesterol (mg/dl)	16.6 ± 3.3	22.1 ± 3.6
HDL-cholesterol (mg/dl)	63.2 ± 2.4	59.2 ± 2.2
LDL-cholesterol (mg/dl)	84.7 ± 3.5	84.2 ± 5.8

Significant different values (Student's *t* test paired data, $P < 0.05$)

The vitamin E concentrations in plasma and lymphocytes as well as the plasma carotenes before and after the cycling stage are presented in Table 4. Plasma vitamin E concentration increased significantly after the cycling stage (12.4%). However, no changes were

Table 1 List of gene-specific primers used in PCR

Genes	Forward primer	Reverse primer	Product size (bp)	GeneBank accession n°
hCu/Zn-SOD	AAGGCCGTGTGCGTGCTGAA	CAAGTCTCCAACATGCCTCT	245	AY450286
hMn-SOD	GAGAAGTACCAGGAGGCGTTG	CAAGCCAACCCCAACCTGAGC	252	BC035422.1
HGP	ACATGCCTACAGGTATGCGT	GAGCAGAACAATTGGACCTA	218	NM_002084
HCAT	TTTGGCTACTTTGAGGTCAC	TCCCCATTTGCATTAACCAG	440	NM_001752
H36B4-rib	ATGTGAAGTCACTGTGCCAG	GTGTAATCCGTCTCCACAGA	420	M17885

Table 4 Changes in lymphocyte vitamin E and in plasma vitamin E and carotenes during the cycling stage

	Before	After
Lymphocytes		
Vitamin E (μM)	512 ± 57	540 ± 40
Plasma		
Vitamin E (μg/ml)	10.5 ± 1.6	11.8 ± 1.8*
Lutein/Zeaxanthin (μg/l)	295 ± 47	283 ± 39
Cryptoxanthin (μg/l)	238 ± 36	236 ± 32
Lycopene (μg/l)	90.4 ± 14.2	88.2 ± 20.5
β-carotene (μg/l)	283 ± 66	273 ± 66

Significant different values (Student's *t* test paired data, $P < 0.05$)

observed in the plasmatic carotene levels. The lymphocyte vitamin E concentration did not change after the cycling stage. The plasma vitamin E concentration was significantly correlated with the serum triglyceride concentrations ($P < 0.001$, $r = 0.708$) and also with the serum cholesterol-VLDL concentrations ($P < 0.001$, $r = 0.704$).

Lymphocyte antioxidant enzyme activities before and after the cycling stage are presented in Table 5. No significant differences were found in lymphocyte catalase and glutathione peroxidase activities. However, a significant increase was observed in total SOD activity (83%). This total SOD activity includes both the CuZn-SOD and the Mn-SOD isoenzyme activities.

The changes in the lymphocyte mRNA levels of the antioxidant enzymes catalase, glutathione peroxidase, CuZn-SOD and Mn-SOD are presented in Table 6. The mRNA levels of catalase and glutathione peroxidase did not change along the study. However, significant increases were observed in the levels of CuZn-SOD and Mn-SOD isoenzymes, correlating to the changes observed in enzyme activity.

Discussion

It has been widely reported that strenuous exercise causes oxidative stress (Alessio 1993; Sjodin et al. 1990; Tauler et al. 2005). The occurrence of oxidative damage during the cycling stage was analysed both in plasma and lymphocytes by determining specific markers and the response of endogenous and exogenous antioxidant systems. The results obtained in plasma showed that the cycling stage induced moderate oxidative stress as it was indicated by the low but significant increases in the levels of plasma carbonyl derivatives and in the test d-Roms. However, the cellular oxidative damage markers, MDA and carbonyl index, analysed in lymphocytes before and after the stage did not show any significant increase, suggesting that changes operating in plasma are not causing intracellular redox unbalance in lymphocyte cells. In addition, no changes were observed in the lymphocyte number after the cycling stage, which could be related with the lower levels of cortisol after the stage. In a previous study developed with very similar subjects, we reported that a mountain cycling stage induced a significant lymphopenia and higher levels of oxidative stress both in plasma and lymphocytes (Tauler et al. 2005), suggesting that exercise intensity could be the main cause for these prominent changes. In fact, mountain cycling stage seems to be a more demanding exercise than the road cycling referred in this study. In this sense, an inverse correlation has been reported between lymphocyte carbonyl derivatives and lymphocyte number (Sureda et al. 2005). This inverse correlation could be related with the activation of lymphocyte apoptotic processes by oxidative stress, resulting in the well-known lymphopenia observed after exhaustive exercises (Mooren et al. 2002; Wang and Huang 2005), such as the mountain cycling stage (Tauler et al. 2005).

Changes in the antioxidant response to the exercise were analysed as well in this study. In agreement with previous findings (Aguilo et al. 2005; Tauler et al. 2005), no significant changes were observed in the plasmatic concentrations of carotenes after the stage. As it has been reported (Aguilo et al. 2005; Tauler et al. 2005), this lack of changes in plasma carotene levels is associated with the maintenance of LDL levels observed after the cycling stage. It this context, the

Table 5 Changes in lymphocyte antioxidant enzyme activities during the cycling stage

	Before	After
Catalase (K/10^9 cells)	17.4 ± 1.7	20.1 ± 1.2
Glutathione Peroxidase (nkat/10^9 cells)	81.9 ± 7.2	104 ± 8
Superoxide Dismutase (pkat/10^9 cells)	11.1 ± 0.5	20.3 ± 1.5*

Significant different values (Student's *t* test paired data, $P < 0.05$)

Table 6 Effects of the cycling stage on the lymphocyte mRNA antioxidant enzyme levels

	Fold induction
Catalase	1.23 ± 0.33
Glutathione Peroxidase	1.69 ± 0.46
CuZn-SOD	2.65 ± 0.58*
Mn-SOD	2.42 ± 0.43*

mRNA levels at the beginning of the stage were arbitrarily referred to as 1. Significant different values (Student's *t* test paired data, $P < 0.05$)

modest plasmatic vitamin E changes could be a consequence of increased lipoprotein turnover (Packer et al. 1989). An increase in plasmatic vitamin E after this cycling stage has been found. This increase is produced by the non-significant increases observed in triglyceride and VLDL levels in plasma after the cycling stage because significant correlations were found between plasma vitamin E and the levels of both triglycerides and cholesterol-VLDL. Furthermore the increases in triglyceride and VLDL-cholesterol levels are higher when the exercise developed is more demanding (Aguilo et al. 2005). Thus, the changes in the serum levels of triglycerides and VLDL-cholesterol could be related directly to exercise intensity and could be used as candidate markers of physical stress induced by a sustained exercise.

Increases in lymphocyte vitamin E after exhaustive exercises have been reported previously (Cases et al. 2005; Tauler et al. 2005). However, lymphocyte vitamin E levels did not change after the present cycling stage. In a previous study, we reported that a mountain cycling stage, that is a more demanding exercise, induced an increase in lymphocyte vitamin E levels, but accompanied by a significant increase in the lymphocyte carbonyl index (Tauler et al. 2005). In the present study, both vitamin E and carbonyl index did not change in lymphocytes after the cycling stage. Altogether, it seems that vitamin E uptake by lymphocytes is activated according to the levels of oxidative stress that the cells are undergoing as a result of exercise intensity with the purpose of protecting the cell against the action of ROS (Cases et al. 2005; Tauler et al. 2005).

The lymphocyte enzymatic antioxidant defences have shown great adaptations to oxidative stress induced by exercise increasing their activities (Tauler et al. 2003, 2005) and also the SOD protein levels (Tauler et al. 2005). It seems that this adaptation depends mostly on the intensity and the duration of the exercise and it is produced after a short deactivating stage (Tauler et al. 2004). In the present work, a significant increase in lymphocyte SOD activity is reported. The most important finding is that this increased activity correlates with significant increases in the mRNA levels of lymphocyte CuZn-SOD and Mn-SOD after the cycling stage. Then, it can be concluded that increased SOD activity was due, at least in part, to the increase in the gene expression. In a similar way, increases in lymphocyte heme oxygenase-1 (HO-1) mRNA expression were found after different exercises (Niess et al. 1999; Thompson et al. 2005). It has been reported that exercise increased the mRNA levels of Mn-SOD in animals (Hollander et al. 2001), which it is thought to be induced by ROS (Gomez-Cabrera et al. 2005) and by factors such as IL-1 and TNF that are increased after exercise (Suzuki et al. 2000). However, it has been suggested that the regulation of the CuZn-SOD isoenzyme is independent of oxidative metabolism and is different from that of Mn-SOD (Suzuki et al. 2000). The exact molecular mechanism operating in our experimental observations are still unknown.

The increase in the lymphocyte vitamin E levels along with the increased SOD mRNA expression could be likely in agreement with the role of ROS as inducers of the early adaptation to oxidative stress. Nevertheless, the data do not exclude other alternative mechanisms. In any case, it has been reported, that especially in the muscle, exercise generates signals important in the cellular physiology (Gomez-Cabrera et al. 2005; McArdle et al. 2005). These signals are essential in the adaptation of the antioxidant defence to the increased oxidative stress induced by exhaustive exercise. It has been shown that the NF-κβ which up-regulates the expression of the antioxidant enzymes, is activated during exercise both in humans (Vider et al. 2001) and in animals (Gomez-Cabrera et al. 2005; Hollander et al. 2001). Recently, it has been reported that ROS generated during exercise are responsible for such activation because inhibition of ROS production prevented the activation of the NF-κβ and therefore the subsequent up-regulation of gene expression of antioxidant enzymes such as Mn-SOD (Gomez-Cabrera et al. 2005).

Fitting with the data presented in this study, it has been previously shown that lymphocyte protein carbonyl derivatives are directly correlated with lymphocyte glutathione peroxidase activity (Sureda et al. 2005). Thus, SOD activation, could play an essential role in the early adaptation to the increased oxidative stress, preventing the initial damage in lymphocytes and the induction of greater antioxidant activation. However, the increase in exercise intensity, i.e. mountain cycling stage, is accompanied by main changes in all antioxidant enzymes and prominent oxidative damage in lymphocytes. Altogether, this suggests that SOD enzymes could act as a primary line of defense against oxidative damage induced by exercise.

In conclusion, the resulting moderate levels of oxidative stress could generate signalling molecules that may be instrumental in inducing subsequent cellular adaptations to counteract the negative effects of the ROS generated by exercise. The nature of such intermediates remains to be further investigated.

Acknowledgments This work has been granted aid by the Spanish Ministry of Health (Programme of Promotion of Biomedical

Research and Health Sciences, Project PI021593), the Spanish Ministry of Science and Education (DEP2005-00238-C04-02/EOU) and the FEDER funding.

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