Oxidative and nitrosative stress in brain mitochondria of diabetic rats

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Abstract

Diabetic encephalopathy, characterized by impaired cognitive functions and neurochemical and structural abnormalities, may involve direct neuronal damage caused by intracellular glucose. The study assesses the direct effect of chronic hyperglycemia on the function of brain mitochondria, the major site of reactive species production, in diabetic streptozotocin (STZ) rats. Oxidative stress plays a central role in diabetic tissue damage. Alongside enhanced reactive oxygen species (ROS) levels, both nitric oxide (NO) levels and mitochondrial nitric oxide synthase expression were found to be increased in mitochondria, whereas glutathione (GSH) peroxidase activity and

manganese superoxide dismutase protein content were reduced. GSH was reduced and GSH disulfide (GSSG) was increased in STZ rats. Oxidative and nitrosative stress, by reducing the activity of complexes III, IV and V of the respiratory chain and decreasing ATP levels, might contribute to mitochondrial dysfunction. In summary, this study offers fresh evidence that, besides the vascular-dependent mechanisms of brain dysfunction, oxidative and nitrosative stress, by damaging brain mitochondria, may cause direct injury of neuronal cells.

Journal of Endocrinology (2005) **187**, 37–44

Introduction

Besides well-described complications, such as autonomic and peripheral neuropathy, type I diabetes mellitus is also associated with gradually developing end-organ damage in the central nervous system (Brands *et al.* 2004). This little-known complication, referred to as ‘diabetic encephalopathy’, is characterized by impairment of cognitive functions and electrophysiologic changes (Allen *et al.* 2004). These functional changes are accompanied by neurochemical and structural abnormalities, as well as by degenerative changes in the brain (Weigner & Jacobsen 1998, McCall 2004). Both micro- and macrovascular cerebral diseases occurring in diabetic patients and the direct neuronal damage caused by chronically elevated intracellular glucose concentrations are implicated in encephalopathy. However, it remains unclear how much of the neuronal impairment is caused directly by intracellular glucose. The direct glucose toxicity in the neurons is especially due to increased intracellular glucose oxidation (Nishikawa *et al.* 2000), which leads to an increase in reactive species production (Bonnefont-Rousselot 2002, Evans *et al.* 2002): in both man and experimentally diabetic rats, oxidative stress seems to play a central role in brain damage (Aragno *et al.* 2000a,b, 2002, Arvanitakis

et al. 2004). Emerging evidence shows that the increased oxidative stress and consequent oxidative damage observed in hyperglycemic conditions begins in the mitochondria, which are the major site of ROS (reactive oxygen species) production (Raha & Robinson 2000, Duchon 2004). Reactive species are byproducts of the mitochondrial respiratory chain that are physiologically counteracted by the intracellular antioxidant systems (Smith *et al.* 1999, Sarkela *et al.* 2001, Green *et al.* 2004). The overproduction of reactive species induced by enhanced glucose oxidation might overwhelm the antioxidant defenses, leading to cell damage. It has been reported that normalizing superoxide mitochondrial production blocks the pathways of hyperglycemic damage (Nishikawa *et al.* 2000). Recently, however, the unique role of brain mitochondrial dysfunction in experimental diabetes has been questioned, and it has been suggested that extramitochondrial factors may cooperate in the induction of oxidative stress associated with type I diabetes (Moreira *et al.* 2004).

This study aimed to characterize the oxidative stress induced by chronic hyperglycemia and its effects on the respiratory chain in the brain mitochondria of diabetic streptozotocin (STZ) rats. Results show that chronic hyperglycemia impairs the mitochondrial respiratory chain, induces oxidative damage through production of

ROS and RNS (reactive nitrogen species) and damages mitochondrial functioning.

Materials and Methods

Animal treatment

Male Wistar rats (Harlan-Italy, Udine, Italy) weighing 200–220 g were cared for in compliance with the Italian Ministry of Health Guidelines (no. 86/609/EEC) and with the *Principles of Laboratory Animal Care* (NIH no. 85–23, revised 1985). They were provided with Piccioni pellet diet (no. 48, Gessate, Milan, Italy) and allowed water *ad libitum*. Hyperglycemia was induced through a single injection of STZ (50 mg/kg) in the tail vein. Three days later, glycemia was measured with *o*-toluidine reagent (Sigma kit, catalog no. 635) on blood collected from the heart (200 µl). Only rats with blood glucose levels of 18–20 mmol/l entered the experimental protocols; normoglycemic rats were used as controls. After 21 days, control and hyperglycemic rats were anesthetized with ether and killed by decapitation after aortic exsanguination. Blood was collected and the plasma isolated. Glycemia was evaluated as described above. The cerebral hemispheres were chiseled and rapidly weighed and homogenized to obtain the mitochondrial fraction.

Isolation of brain mitochondria

Mitochondria were extracted from brain homogenates of normal and STZ rats by the method described by Morin *et al.* (2001). The homogenates (17%, w/v) were centrifuged at 2000 *g* for 3 min. The supernatant A was preserved and the pellet diluted in 20 ml buffer A (20 mM Tris-HCl, 250 mM sucrose, 40 mM KCl, 2 mM EGTA and 1 mg/ml BSA, pH 7.2, at 4 °C) and centrifuged at 2000 *g* for 3 min. Supernatant B was added to supernatant A and centrifuged at 12 000 *g* for 10 min. The pellet obtained was resuspended in 20 ml of 15% (v/v) Percoll, and 3 ml of this mixture were laid on two preformed layers of 3.5 ml of 23% (v/v) Percoll and 3.5 ml of 40% (v/v) Percoll. The gradient was centrifuged for 5 min at 30 700 *g*. The fraction between the two gradient layers was collected and diluted 1:4 with buffer A. The suspension was centrifuged twice at 12 000 *g* for 10 min, and the pellet (mitochondria-enriched fraction) was resuspended in 300 µl buffer B (300 mM mannitol, 10 mM KH₂PO₄, 19 mM KCl and 5 mM MgCl₂, pH 7.2) at 4 °C.

Detection of nitric oxide (NO): total nitrite and oxidation of oxyhemoglobin

Total nitrite concentration in fresh mitochondria was used as an indicator of nitric oxide (NO) synthesis. Nitrates in samples were stoichiometrically reduced to nitrites by

incubating 250 µl sample for 15 min at 37 °C, in the presence of 1 IU/ml nitrate reductase, 500 µM NADPH and 50 µM FAD in a final volume of 400 µl. When nitrate reduction was complete, unused NADPH, which interferes with subsequent nitrite determination, was oxidized with 100 IU/ml lactate dehydrogenase and 100 mM sodium pyruvate in a final reaction volume of 500 µl and incubated for 5 min at 37 °C (Millar & Thiemeermann 1997). Subsequently, total nitrites were assayed by adding 500 µl Griess reagent (4% sulfanilamide and 0.2% naphthylendiamide in 10% phosphoric acid) to each sample (Green *et al.* 1981).

Production of NO was measured in fresh brain mitochondria by monitoring the oxidation of oxyhemoglobin to methemoglobin spectrophotometrically at 37 °C in a reaction medium containing 50 mM phosphate buffer, 1 mM CaCl₂, 1 mM L-arginine, 100 µM NADPH, 10 µM dithiotreitol, 4 µM superoxide dismutase (SOD), 0.1 µM catalase, mitochondria (1.0 mg prot/ml), and 5 µM oxyhemoglobin (expressed per heme group). The kinetics was monitored at 577 nm ($\epsilon = 11.2$ mM/cm), and absorbance changes were expressed as nmol NO/min per mg prot. Control experiments adding 1 mM NG-methyl-L-arginine (L-NMMA) were performed to confirm that hemoglobin oxidation occurred only by NO formation. Addition of L-NMMA inhibited the rate of hemoglobin oxidation by 90% (Arnaiz *et al.* 1999).

Western blot analysis

Mitochondrial nitric oxide synthase (MtNOS) and manganese superoxide dismutase (MnSOD) were detected on fresh mitochondria by Laemmli's (1970) method. Aliquots of sample containing ≈ 30 µg proteins were resolved respectively on 7.5% and 10% SDS-polyacrylamide gel, and then blotted onto nitrocellulose membranes (Amersham Biosciences, Braunschweig, Germany). The membranes were blocked with 5% (w/v) nonfat dry milk in 5 mM Tris-HCl, pH 7.4, containing 200 mM NaCl and 0.05% (v/v) Tween 20 (TBS-Tween) for 1 h at 25 °C and then incubated with mouse monoclonal antibody against mtNOS diluted 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit polyclonal antibody against MnSOD diluted 1:10 000 (Upstate, Lake Placid, NY, USA). The membranes were incubated with appropriate peroxidase-labeled secondary antibody prepared in TBS-Tween containing 2% (w/v) nonfat dry milk. Immunoreactive proteins were detected with a chemiluminescence assay (ECL, Amersham) and subsequent exposure to film for 2–10 min. Specific bands were quantified by densitometry using analytic software (Bio-Rad, Multi-Analyst, Munich, Germany).

Pro-oxidant and antioxidant compounds

The pro-oxidant state was determined by monitoring the generation of hydrogen peroxide (H₂O₂) after adding

100 µl sample at 30 °C to a cuvette containing a respiration buffer: 7.4 IU horseradish peroxidase, 40 µM acetylated ferrocyanochrome-*c* and 5 µM *p*-hydroxyphenylacetic acid. H₂O₂ release was evaluated as the increase of acetylated ferrocyanochrome-*c* oxidation rate, and monitored at 550 minus 540 nm with an absorption coefficient of 19.9 mmol/l per cm, as described by Zoccarato *et al.* (1993).

The glutathione (GSH)/glutathione disulfide (GSSG) ratio was evaluated by the Owens and Belcher (1965) method. A mixture was directly prepared in a cuvette: 0.05 M Na-phosphate buffer, pH 7.0; 1 mM EDTA, pH 7.0; and 10 mM DTNB plus an aliquot of the sample. GSH content was evaluated after 2 min at 412 nm and expressed as µg/mg prot. Suitable volumes of diluted GSH reductase and of NADPH were then added to evaluate the total GSH level. The difference between total GSH and GSH content represents the GSSG content (expressed as µg/mg prot); the ratio between GSSG content and GSH is considered a good measure of antioxidant status.

GSH peroxidase activity was determined on freeze-thawed and sonicated samples. Mitochondria were suspended in 1 ml buffer (50 mM Tris-HCl, pH 7.6; 5 mM EDTA; 1 mM GSH; 0.22 mM NaDPH; and 0.4 U of GSH reductase), and the reaction was initiated by adding tert-butyl-hydroperoxide to a final concentration of 0.22 mM. NADPH consumption was monitored continuously over 3 min at 340 nm in a spectrophotometer. Final results are expressed as units/mg protein of mitochondrial protein (Yang *et al.* 2000).

Mitochondrial complexes

Mitochondrial complex II was measured as the rate of cytochrome *c* reduction at 550 nm and 37 °C (Morin *et al.* 2002). The reaction mixture (1 ml) contained the following: 10 mM Tris-HCl, pH 7.2; 0.2 mM ADP; 3 µM rotenone; 0.3 mM KCN; 10 mM succinate; and 40 µg mitochondrial protein (samples were freeze-thawed and sonicated). The reaction was initiated by adding 40 µM cytochrome *c* to the cuvette. After 1 min, 10 mM malonate were added, and the inhibited rate was measured for 1 min, after which 50 µM succinate were added, and the rate was measured again for 1 min.

Mitochondrial complex III (cytochrome *c* reductase) was measured as the rate of cytochrome *c* reduction at 550 nm and 37 °C (Morin *et al.* 2002). The reaction mixture (1 ml) contained the following: 10 mM Tris-HCl, pH 7.2; 0.2 mM ADP; 3 µM rotenone; 0.3 mM KCN; 10 mM succinate; and 40 µg mitochondrial protein (samples were freeze-thawed and sonicated). The reaction was initiated by adding 40 µM cytochrome *c* to the cuvette. After 1 min, 10 mM malonate were added, and the inhibited rate was measured for 1 min, after which 50 µM decylubiquinol were added, and the rate was measured again for 1 min.

Mitochondrial complex IV (cytochrome *c* oxidase) was measured as the rate of cytochrome *c* oxidation at 550 nm and 37 °C (Morin *et al.* 2002). The reaction mixture in the cuvette contained the following: 10 mM potassium phosphate buffer, pH 7.2, and 40 µM reduced cytochrome *c*. The reaction was initiated by the addition of 40 µg mitochondrial protein (samples were freeze-thawed and sonicated), and the rate was measured for 1 min.

Mitochondrial complex V activity (ATP synthase) was measured as the hydrolysis rate of ATP to ADP plus inorganic phosphate (Pi), as described by Morin *et al.* (2002). Freeze-thawed and sonicated mitochondria (50 µg protein) were incubated in 500 µl ATPase buffer (50 mM Tris and 5 mM MgCl₂, pH 7.5) at 37 °C with 5 mM ATP for 10 min. The reaction was halted by adding 500 µl of 10% (w/v) trichloroacetic acid. Each assay was centrifuged at 3000 *g* for 20 min, and 500 µl supernatant were mixed with 500 µl water. Pi production was measured as described by Fiske and Subbarow (1925), and 1.6 ml of 10.1 mM ammonium molybdate in 0.42 M sulfuric acid were added. The reaction measuring the Pi production was started by adding 400 µl of a mixture containing 0.72 M sodium bisulfite, 41.6 mM sodium sulfite and 11.0 mM 4-amino-3-hydroxy-1-naphthalenesulfonic acid. After 10-min incubation at room temperature, the Pi production was monitored at 660 nm.

Mitochondrial damage

Cytochrome *c* release was measured in the cytosol fraction obtained by centrifugation during mitochondria isolation, as described by Perez-Pinzon *et al.* (1999). Briefly, 1 ml cytosol was transferred to a spectrophotometer cuvette, and the absorption peak was measured at 419 nm. The final cytochrome *c* concentration was obtained from a standard curve and was expressed as nmol/mg protein.

ATP concentration was determined by Debetto *et al.*'s (1982) method: 3.5% (v/v) perchloric acid was added to an aliquot of sample and immediately cooled on ice for 40 min. The acid supernatant was neutralized with K₂CO₃ (0.5 M) and the suspension centrifuged to remove insoluble material. The sample (10 µl) was injected into the HPLC column; eluent: potassium phosphate buffer 0.1 M, pH 5.3; column: symmetry C18 (Waters column 5 µm, 3.9 × 150 mm).

Statistical analysis

All results are presented as means ± S.D. Differences between means were analyzed for significance by Student's *t*-test (Matthews & Farewell 1988).

Results

Biochemical parameters

The body weight of STZ diabetic rats was significantly lower than that of controls 21 days after injection of STZ

(control group: 250.9 ± 12.4 g; STZ: 224.7 ± 10.4 g). STZ was rapidly eliminated from the body: about 80% appeared in the urine within 6 h (Oberley 1988). Blood glucose levels in STZ-treated rats 3 days after STZ-injection were 18–20 mmol/l. The glucose level remained unchanged during the 21-day period. At the end of the protocol (21 days), the glycemia level was 6.04 ± 0.64 mmol/l in the control group and 20.72 ± 2.25 mmol/l in the STZ-treated group.

NO synthesis

Figure 1 illustrates the levels of NO in the enriched fraction of mitochondria isolated from the brain of normal and diabetic rats. Panel A shows the enhanced level of total nitrite level (NO_2/NO_3) in STZ-rats versus control rats. NO_2/NO_3 are the primary oxidation products of NO after reaction with oxygen, and thus the nitrite-plus-nitrate concentration is usually employed as indicator of NO synthesis (Green *et al.* 1981). Panel B shows NO production evaluated in terms of oxidation of oxymyoglobin to methemoglobin. In STZ rats, the production of NO was more than double that of the control group. Panel C reports a representative experiment showing that mtNOS in STZ rats appeared to be more expressed than in controls. All isoforms of NOS may be expressed in the brain. Brain mtNOS was identified mainly as nNOS, which can be regulated by a variety of stimuli and by several physiologic conditions (Cooper 2002). The mtNOS isoform is expressed constitutively and has been located in the inner mitochondrial membrane (Giulivi *et al.* 1998).

Antioxidant and pro-oxidant levels

GSSG/GSH ratio, MnSOD and H_2O_2 levels were determined in the enriched mitochondrial fraction (Fig. 2). The ratio GSSG/GSH (panel A) was increased, since the GSSG level was increased and the GSH level reduced in STZ rats versus controls. The content of MnSOD (panel B), a specific mitochondrial antioxidant enzyme, was markedly lower in STZ rats. Moreover, a marked increase of hydrogen peroxide, the product of dismutation of superoxide by MnSOD, was observed (panel C), further indicating that radical species overproduction consumes endogenous antioxidants and overwhelms the antioxidant capability. The activity of GSH peroxidase was also reduced in the mitochondrial fraction of diabetic rats versus controls (Table 1).

Respiratory chain

The activity of complex II, III, IV and V is shown in Table 1. The complex II did not show significant change versus controls. Complex III activity (cytochrome *c*

Figure 1 Effect of STZ-induced diabetes on NO level. (A) NO_2/NO_3 level; (B) NO production; (C) representative Western blot showing mtNOS levels. The histogram shows the band densities of 6–7 experiments compared with relative β -actin band densities and expressed as percent variation versus the control group. Data are means $\pm$ s.d. of 6–7 rats per group. * $P < 0.05$, statistically significant versus control rats.

reductase) fell from 66.72 ± 13.97 in control rats to 31.19 ± 5.09 in STZ rats (50% lower). Complex IV

A **B** **C**

Figure 2 Effect of STZ-induced diabetes on antioxidant and pro-oxidant levels. (A) GSSG/GSH ratio; (B) representative Western blot showing MnSOD levels. The histogram shows the band densities of 6–7 experiments compared with relative β -actin band densities and expressed as percent variation versus the control group. (C) H_2O_2 levels. Data are means $\pm$ S.D. of 6–7 rats per group. * $P < 0.05$, statistically significant versus controls.

(cytochrome *c* oxidase) and complex V (ATP synthase) activities were also significantly reduced (by 30%) in diabetic rats versus controls.

Table 1 Effect of STZ-induced diabetes on respiratory mitochondrial enzymatic activities and on antioxidant enzyme activity

	CONTROL	STZ
Complex II (nmol/min/mg protein)	355.45 $\pm$ 36.41	443.65 $\pm$ 65.54
Complex III–Cyt c-reductase (nmol/min/mg protein)	66.72 $\pm$ 13.97	31.19 $\pm$ 5.09*
Complex IV–Cyt c-oxidase (nmol/min/mg protein)	213.56 $\pm$ 14.69	160.56 $\pm$ 33.57*
Complex V–ATP-synthase (nmol Pi/min/mg protein)	2.79 $\pm$ 0.18	1.79 $\pm$ 0.25*
GSH peroxidase (U/mg protein)	14.66 $\pm$ 0.93	10.31 $\pm$ 1.40*

Data are mean $\pm$ S.D. of 6–7 rats per group

* $P < 0.05$, statistically significant versus control rats.

Mitochondrial function

To evaluate the entity of mitochondrial dysfunction, ATP level and cytochrome *c* release in the cytosol were measured. ATP levels in mitochondria of STZ rats were decreased versus the control group (Fig. 3, panel A), and the cytochrome *c* level in the cytosol was increased (Fig. 3, panel B). Release of cytochrome *c* into the cytosol from mitochondria is considered a good marker of mitochondrial membrane damage (Perez-Pinzon *et al.* 1999).

Discussion

The study confirms that mitochondria isolated from the brain of hyperglycemic rats show increased ROS levels, associated with reduced antioxidant barriers, in terms of GSSG/GSH ratio, GSH peroxidase activity and MnSOD content. Moreover, besides the enhanced level of ROS, NO levels are also increased, and expression of mtNOS appears to be significantly increased in the brain mitochondria of diabetic rats. NO is a diffusible regulatory molecule involved in a wide range of physiologic and pathologic events (Sarkela *et al.* 2001). Originally known as endothelial relaxation factor, it has been shown to be an intracellular messenger in physiologic processes such as regulation of blood flow and neurotransmission (Brown 1999). The borderline between the physiologic and pathologic activities of NO is still a matter of controversy, but it is generally accepted that both its concentration and subcellular production site are critical in determining whether it acts as a signaling or a neurotoxic molecule. It now appears certain that, in neurons, NO is generated in mitochondria by NO synthase (NOS) in the inner membrane of the mitochondria (Giulivi *et al.* 1998). NO, at low and physiologic concentrations, reversibly binds to the Cu_B center of cytochrome *c* oxidase (complex IV) and can partially inhibit electron transfer to O_2 , acting in competition with oxygen (50–100 nM NO inhibits 50%

Figure 3 Effect of STZ-induced diabetes on ATP concentration (A) and cytochrome *c* release (B). Data are means $\pm$ s.d. of 6–7 rats per group. * $P < 0.05$, statistically significant versus controls.

of cytochrome *c* oxidase activity) (Moncada & Erusalimsky 2002). At those concentrations, NO sets up cytochrome *c* oxidase activity and participates in maintaining radical matrix steady state.

In diabetic rat brain mitochondria, we show here that NOS activity increases, with consequent NO hyperproduction: in those conditions, NO inhibits cytochrome *c* oxidase activity but might also act on other mitochondrial components, inhibiting other respiratory

chain complexes by nitrosilation or oxidizing protein thiols (Clancy *et al.* 1994). In line with this finding, we observed a reduction in the activities of complex III (cytochrome *c* reductase), IV (cytochrome *c* oxidase) and V (ATP synthase) of the respiratory chain, leading to the impairment of the cell energy state. Beside its effects on the respiratory chain, NO may also contribute to cell damage in diabetes, modulating glucose entry into the cells. Indeed, NO has been shown to upregulate glucose transporters in neurons (Bolaños *et al.* 2004). This activity has been regarded as protective in conditions (such as cerebral ischemia) in which the glucose supply to the brain is reduced (Friberg *et al.* 2002). Conversely, the upregulation of glucose transporters by NO might be detrimental in conditions characterized by excessive glucose supply, such as diabetes, in which increased intracellular glucose leads to an oversupply of electrons in the mitochondrial transfer chain, resulting in mitochondrial membrane hyperpolarization and a further increase in free-radical production.

On the other hand, altered NOS expression and activity increase peroxynitrite production, which overwhelms the detoxifying reactions so that the effects mediated by NO-derived reactive species prevail. Indeed, peroxynitrite, a harmful oxidant formed by reaction between superoxide and NO, reacts with a variety of molecules, including protein and non-protein-thiols, unsaturated fatty acids and DNA, thus affecting energy conservation mechanisms and oxidative post-translation modification of protein, and ultimately causing neuronal cell death (Murray *et al.* 2003).

Besides its direct detrimental effect on mitochondrial function, RNS interplays with the simultaneous oxidative stress, amplifying mitochondrial damage. Indeed, peroxynitrite can inhibit MnSOD, thereby reducing its quenching activity on superoxide. Here we show that MnSOD content is reduced in mitochondria, suggesting that the dismutation of superoxide, overproduced as a result of glucose-induced oxidative stress, might be impaired. The role of mitochondrial MnSOD is critical in protecting neurons from free-radical damage: mutations which affect SOD, with consequent increase of reactive species, have been reported in a number of neurologic diseases (Raha *et al.* 2000). The increased H_2O_2 is related to the drastic loss of GSH, which is required to activate GSH peroxidase, the H_2O_2 detoxifying enzyme. In fact, we also observed the activity of GSH peroxidase to be clearly affected in STZ rats. However, GSH loss might to be partially dependent on the opening of the mitochondrial permeability transition pore (Moreira *et al.* 2005), as suggested by the observation of a higher release of cytochrome *c* in mitochondria isolated from STZ rats. The mitochondrial pool of GSH is considered vital for cell survival: after severe mitochondrial GSH depletion, cell death occurs; this, however, is delayed by antioxidant treatment (Han *et al.* 1997, 2003).

Moreover, the brain mitochondria of diabetic rats show consistently reduced ATP levels, as a result of the prominent changes in activity of the mitochondrial respiratory chain enzymes in such rats. ATP content is closely related to maintenance of membrane potential; thus, a reduction in ATP would produce an irreversible drop in membrane potential, opening the permeability transition pore and triggering mechanisms of cell death. Indeed, we also observed increased cytochrome *c* release into the cytosol. Cytochrome *c* is a marker of mitochondrial dysfunction (Yang *et al.* 1997) and a cell-death trigger activator that operates through activation of specific caspases and consequent induction of apoptosis (Kluck *et al.* 1997).

In brief, this study offers fresh evidence that oxidative stress and nitrosative stress cooperate in damaging the brain mitochondria of diabetic rats. These findings may account for the direct injury of neuronal cells that is added to the micro- and macrovascular damage. Further studies are needed to establish whether the damage to mitochondria causes or exacerbates diabetic encephalopathy.

Acknowledgements

This work was supported in part by FIRB-2001, MIUR (Ministero dell'Istruzione, Università e Ricerca) and the Regione Piemonte, Italy. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 19 June 2005

Accepted 1 July 2005