Hyperglycemia induces apoptosis in rat liver through the increase of hydroxyl radical: new insights into the insulin effect

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Abstract

In this study, we analyzed the contribution of hydroxyl radical in the liver apoptosis mediated by hyperglycemia through the Bax–caspase pathway and the effects of insulin protection against the apoptosis induced by hyperglycemia. Male adult Wistar rats were randomized in three groups: control (C) (sodium citrate buffer, i.p.), streptozotocin (STZ)-induced diabetic (SID) (STZ 60 mg/kg body weight, i.p.), and insulin-treated SID (SID + I; 15 days post STZ injection, SID received insulin s.c., twice a day, 15 days). Rats were autopsied on day 30. In liver tissue, diabetes promoted a significant increase in hydroxyl radical production which correlated with lipid peroxidation (LPO) levels. Besides, hyperglycemia significantly increased mitochondrial BAX protein expression, cytosolic cytochrome *c* levels, and caspase-3 activity leading to an increase in apoptotic index. Interestingly, the treatment of diabetic rats with desferoxamine or tempol (antioxidants/hydroxyl radical scavengers) significantly attenuated the

increase in both hydroxyl radical production and in LPO produced by hyperglycemia, preventing apoptosis by reduction of mitochondrial BAX and cytosolic cytochrome *c* levels. Insulin treatment showed similar results. The finding that co-administration of antioxidants/hydroxyl radical scavengers together with insulin did not provide any additional benefit compared with those obtained using either inhibitors or insulin alone shows that it is likely that insulin prevents oxidative stress by reducing the effects of hydroxyl radicals. Importantly, insulin significantly increased apoptosis inhibitor protein expression by induction of its mRNA. Taken together, our studies support that, at least in part, the hydroxyl radical acts as a reactive intermediate, which leads to liver apoptosis in a model of STZ-mediated hyperglycemia. A new anti-apoptosis signal for insulin is shown, given by an increase of apoptosis inhibitor protein.

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Introduction

Diabetes is a common metabolic disorder in humans, which is associated with significant morbidity and mortality, and is a contributor to the development of other diseases. Indirectly or directly, the liver is a major target of insulin action. The onset of diabetes is accompanied by development of major biochemical and functional abnormalities in the liver, including alterations in carbohydrate, lipid, and protein metabolism, and changes in antioxidant status (McLennan *et al.* 1991, Saxena *et al.* 1993, Chatila & West 1996, Harrison *et al.* 2006). The prevalence of hepatobiliary diseases is increased in patients with either type 1 or type 2 diabetes (Saxena *et al.* 1993, Bell & Allbright 2007). Even with insulin treatment, diabetic patients show profound disturbances in tissue growth (Porte & Schwartz 1996). Clinically, altered

liver size is seen in both juvenile and adult diabetic patients, which can be the result of alteration in cell number, cell growth, and/or cell death (apoptosis; Chatila & West 1996, Marangiello & Giorgetti 1996).

West indicated an increase in oxidative damage in both type 1 and type 2 diabetes as well as deficits in antioxidant defence enzymes and vitamins. It is argued that oxygen, antioxidant defences, and cellular redox status should be regarded as central players in diabetes (West 2000). Laaksonen *et al.* reported increased lipid peroxidation (LPO) in plasma of young men with type 1 diabetes using the malondialdehyde (MDA) test. MDA is formed when polyunsaturated fatty acyl chains are attacked by hydroxyl radicals, which can also damage DNA-generating characteristic products, i.e. 8-hydroxy-2 deoxyguanosine (Laaksonen *et al.* 1996). There is accumulating evidence especially in diabetic animal models

(such as streptozotocin (STZ)-induced diabetic (SID) rats and mice) that hyperglycemia induces acceleration of hydroxyl radical generation and this has been correlated with the level of thiobarbituric acid (TBA) – reactive compounds as an estimation of LPO (Ohkuwa *et al.* 1995, Winiarska *et al.* 2004).

On the other hand, it was established that hyperglycemia increases mitochondrial reactive oxygen species (ROS) production, which could represent a key event in the development of diabetes complications (Nishikawa *et al.* 2000, Kiritoshi *et al.* 2003). The initial cellular response to high glucose challenge is the generation of ROS, which rapidly induces apoptotic cell death (Park *et al.* 2001).

It is known that high D-glucose induces endothelial apoptosis through activation of the Bax–caspase proteases pathway. The effectors of apoptosis are now well known to be represented by a family of intracellular cysteine proteases known as caspases (Green & Reed 1998). A feature of apoptosis that impinges on caspases is altered mitochondrial function characterized by a reduction in the electrochemical gradient across the mitochondrial membrane and release of mitochondrial cytochrome *c* to cytoplasm (Liu *et al.* 1996), and it is inhibited by the presence of Bcl-xL in these organelles (Fraser & Evan 1996, Yang *et al.* 1997). Translocation of pro-apoptotic BAX protein into the mitochondrial membrane is accompanied by a significant increase in caspase-3 (CASP3) and caspase-9 (CASP9) activities (Nakagami *et al.* 2002).

Several studies have been carried out to evaluate the alterations in cell mitochondrial functions from brain, heart, liver, and kidney of diabetic rats (Moreira *et al.* 2006). However, results are sometimes controversial, since experimental conditions such as age and strain of used animals are diverse.

In view of the above lines of evidence, the objective of the present study was to investigate whether hyperglycemia produces an increase in hydroxyl radical, which exerts a regulatory action on apoptosis in liver through the Bax–caspase pathway. In addition, the effect of insulin over this pathway was also evaluated.

Materials and Methods

Animals and treatments

Experiments were performed in 100-day-old male Wistar rats. They were housed two per cage and maintained in a room at constant temperature with a 12 h light:12 h darkness cycle. Animals were fed with standard pellet diet and water made available *ad libitum*. All the experimental protocols were performed according to the Regulation for the Care and Use of Laboratory Animals (Expedient 6109/012 E.C. Resolution 267/02) and approved by the Institutional Animal Use Committee of the National University of Rosario, Argentina.

SID was induced by a single dose of STZ (60 mg/kg body weight (bw), i.p., in 50 mM citrate buffer, pH 4.5; Ha *et al.* 1994). Rats of the control group (C) ($n=6$) received an i.p. injection of an equal volume of citrate buffer. It is known that STZ is excreted within 48 h from injection and therefore cannot be a direct effect of oxidative stress (Karunanayake *et al.* 1974). Fifteen days after STZ injection, a time when the toxic effect of the drug on the liver would have disappeared (Carnovale & Rodriguez Garay 1984, Carnovale *et al.* 1986), blood samples were obtained from the tail vein, and glucose concentrations were measured by means of a Surestep glucometer (Glucostix, Bayer Health-Care). Successful induction of diabetes was defined as a blood glucose level of >13.2 mmol/L. On day 15 post STZ treatment, SID rats were randomly assigned to two groups ($n=6$ each group): diabetic animals (SID) and insulin-treated diabetic animals (SID+I). Insulin (Betasint insulin, BETA S.A. Laboratories, Buenos Aires, Argentina) was administered s.c. twice a day (at 0800 and 2000 h) until the animals were killed. Blood glucose levels were measured every day, each time just before insulin injection. The doses of insulin were adjusted to reach target blood glucose levels of 6–9 mmol/L. Between 1000 and 1200 h on day 30, rats were weighed, anesthetized with sodium pentobarbital solution (50 mg/kg bw, i.p.), and killed. Blood was obtained by cardiac puncture. Plasma was separated by centrifugation (2200 *g* for 5 min at 4 °C). Liver was promptly removed, and slices of liver tissue were processed for immunohistochemical studies. Remaining liver tissue was stored at -70 °C until the analytical assays were performed.

For hydroxyl radical detection, other four rats belonging to each different group under study (C, SID, SID+I, SID+desferoxamine (DES), SID+DES+I, SID+tempol (TEM), and SID+TEM+I) were injected with trapping agent salicylic acid (SA; 100 mg/kg bw, i.p.) 30 min before the animals were killed, and the livers were removed.

For studies on inhibition of hydroxyl radical production, after 15 days of diabetes, STZ-induced diabetes rats were separated into different groups and received different hydroxyl radical inhibitors alone or co-administered with insulin. Each group contained at least six animals. DES is an iron chelator that prevents the formation of hydroxyl radicals from hydrogen peroxide via inhibition of the Fenton and Haber–Weiss reactions (Kaur & Halliwell 1994). DES (100 mg/kg bw, i.p.) was administered to rats, once a day, in saline solution starting 15 days after injection of STZ and for 15 days. TEM is a stable piperidine nitroxide that permeates biological membranes, and reduces the formation or the effects of hydroxyl radicals by scavenging superoxide anions or by reducing intracellular Fe^{+2} concentrations or by directly scavenging hydroxyl radicals (Chatterjee *et al.* 2000). TEM (20 mg/kg bw, i.v.) was administered to rats, once a day, in saline solution starting 15 days after injection of STZ and for 15 days.

Hydroxyl radical ($\cdot\text{OH}$) detection

The *in vivo* measurement of $\cdot\text{OH}$, a highly reactive free radical, is very difficult (Kaur & Halliwell 1994). Thus, salicylate has been used as a trapping agent for detecting $\cdot\text{OH}$ *in vivo* (Grootveld & Halliwell 1986, Pryor & Godber 1991). Attack by $\cdot\text{OH}$ upon salicylate produces 2,3-dihydroxybenzoic acid (2,3-DHBA), 2,5-DHBA, and catechol. Among these hydroxylated products of salicylate, 2,3-DHBA is reported to be solely formed by direct $\cdot\text{OH}$ attack. As a result, the method of salicylate hydroxylation is useful in predicting the occurrence of oxidative stress *in vivo*, and the measurement of 2,3-DHBA has been proposed as a marker for the assessment of oxidative stress (Pryor & Godber 1991). For this experiment, 30 min after SA injection (100 mg/kg bw, i.p.), the rats were anesthetized with sodium pentobarbital (50 mg/kg bw) and killed.

SA and 2,3-DHBA were measured according to the methods of Tsai *et al.* (1998) and Yamamoto *et al.* (2001) respectively, both with modifications. For the determination of SA and 2,3-DHBA, tissue samples (within 0.5 g) were homogenized in ice-cold saline (0.5 ml), and deproteinized by the addition of 10% v/v perchloric acid containing 1 mM EDTA and 100 μM sodium pyrosulfite. Hydrochloride (1 M, 0.4 ml) was added to the supernatant fraction obtained by centrifugation. The resulting solution was extracted with 10 ml of diethyl ether by mixing thoroughly for 1 min and was then centrifuged. The organic phase was collected, evaporated to dryness under nitrogen, and dissolved in 300 μl mobile phase (30 mM sodium citrate/27.7 mM sodium acetate, pH 2.80). According to the method, the samples were analyzed using a Waters HPLC system, equipped with a Model 1515 pump, and Waters 2465 electrochemical detector (ECD; Waters, Milford, MA, USA). Reverse-phase HPLC analysis was carried out using a Luna 5 μm C18(2) 100A column (250 $\times$ 4.6 mm, Phenomenex, Torrance, CA, USA). The glassy carbon-working electrode was set at a potential of +0.85 V, and the flow rate was 0.6 ml/min. No interfering peaks were detected in blank samples. To determine SA levels, an u.v. detector was coupled to the system monitoring absorbance at 295 nm. The ratio of 2,3-DHBA to SA was obtained.

Assays for lipid peroxidation

LPO levels were determined as indirect measurements of ROS production. The amount of aldehydic products generated by LPO in total homogenate fractions was quantified by the TBA reaction according to the method of Ohkawa *et al.* (1979) and measured by HPLC with modifications introduced by Young & Trimble (1991). The amount of TBA reactants (TBARS) was expressed in terms of MDA using 1,1,3,3-tetramethoxypropane as a standard. Protein content was measured by the method of Lowry *et al.* (1951) using BSA as a standard. Results were expressed as nmol of MDA per mg of protein.

Analysis of protein levels by western blotting

Western blotting was performed for the analysis of AKT, phosphorylated AKT (P-AKT), phosphoinositol-3 kinase (PI3K), BAD, BAX, Bcl-xL (BCL2L1), X-linked inhibitor of apoptosis protein (XIAP), and cytochrome *c* proteins. Briefly, mitochondria-enriched fractions were prepared from liver tissues that were homogenized in four volumes of 0.150 M KCl with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 $\mu\text{g}/\text{ml}$ leupeptin, and 1 $\mu\text{g}/\text{ml}$ aprotinin). Homogenates were centrifuged at 1000 *g* to remove unbroken cells, nuclei, and heavy membranes. Mitochondria-enriched fractions were then obtained by the centrifugation of supernatant at 6000 *g* at 4 °C for 15 min. Then, the supernatant was centrifuged at 45 000 *g* for 1 h to obtain the cytosolic fraction (Ronco *et al.* 2004). Proteins were quantified according to Lowry *et al.* (1951). For detection, 25 μg protein was subjected to 12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (PerkinElmer Life Sciences, Boston, MA, USA). After blocking, blots were incubated overnight at 4 °C with monoclonal primary antibodies (AKT and P-AKT (1:600, Cell Signaling, Danvers, MA, USA); PI3K p85 α , BAD, BAX, BCL-XL, XIAP, and cytochrome *c* (1:600, Santa Cruz Biotechnology, Santa Cruz, CA, USA)). After this, membranes were incubated with secondary antibodies such as IgG-peroxidase conjugates (1:5000, Amersham Life Science), and the resulting bands were detected by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) detection. Autoradiographs were obtained by exposing PVDF membranes to Kodak XAR film, and the bands were quantified by densitometry (Shimadzu CS-9000).

Determination of apoptosis

Caspase-3 activity and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assays were performed as quantitative indexes of apoptosis.

Caspase-3 activity assay

The activity of caspase-3 was determined according to the manufacturer's instructions using an EnzChek caspase-3 assay kit (Molecular Probes, Eugene, OR, USA). The tissues were homogenized in lysis buffer (10 mM Tris, 200 mM NaCl, 1 mM EDTA, and 0.001% Triton X-100). Subsequently, after differential centrifugation, the cytosolic fraction from each sample was mixed with Z-Asp-Glu-Val-Asp-AMC substrate solution. A standard curve of AMC ranging from 0 to 100 μM was run with each set of samples. A control sample without enzyme was used in each assay to determine the background fluorescence of the substrate. As an additional control, 1 μl of the 1 mM Ac-Asp-Glu-Val-Asp-CHO (aldehyde), the caspase-3 inhibitor stock solution, was added. Fluorescence was

measured at an excitation wavelength of 360 nm and an emission wavelength of 465 nm in a DTX 880 Multimode Detector (Beckman Coulter, Brea, CA, USA).

Determination of apoptotic index

An apoptosis detection system was utilized which catalytically incorporates fluorescein-12-dUTP at the 3'-OH DNA ends using the principle of the TUNEL assay, with direct visualization of the labeled DNA (Promega). Light microscopic analysis of hematoxylin- and eosin-stained slides was used to quantify apoptotic cells, which were identified by morphological criteria (increased eosinophilic cytoplasm, darkened nucleus, and pycnotic separation of cytoplasmic membrane from neighboring cells). To corroborate the incidence of apoptotic bodies, serial sections were stained with hematoxylin-eosin. An apoptotic index (AI) was calculated for each sample by counting the number of positively stained hepatocyte nuclei divided by the total number of hepatocytes and expressed as percentage. The number of apoptotic hepatocytes was assessed by systematically scoring at least 10 000 hepatocytes per slide at a magnification of 400× (Gold *et al.* 1994, Klasinguti *et al.* 2000).

Measurement of Xiap mRNA levels or RNA isolation and reverse transcription-PCR

Total RNA was isolated using the TRIzol method (Life Technologies). RNA was dissolved in RNase-free water and kept at -80 °C until use. Reverse transcription (RT) of 3 µg total RNA was performed with oligo(dT) primer, and cDNA samples were stored at -20 °C until assayed. The primers used were *Xiap* (Bio-Synthesis, Lewisville, TX, USA; sense 5'-TCTGGTGTGAGTTCA-GATAGG-3'; antisense 5'-TGGATACCACTTAGCAT-GCTG-3') and β -actin (Genset Oligos; sense 5'-GGT-GACGAGGCCAGAGCAAG-3'; antisense 5'-GATCCA-CATCTGCTGGAAGGT-3'). The PCR was performed in a GenAmp PCR System 2400 thermocycler (Perkin Elmer) using the following conditions: 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and a final extension phase of 72 °C for 10 min. The number of thermal cycles used was 36. RT-PCR products were then resolved on a 1.5% agarose gel, and bands were visualized using a High Performance Ultraviolet Transilluminator (UVP, Upland, CA, USA). Images of the RT-PCR ethidium bromide-stained agarose gels were acquired, and quantification of the optical density

(OD) of bands was performed using the Gel-Pro Analyzer (Media Cybernetics, Bethesda, MD, USA) software (Ronco *et al.* 2009). Results were expressed as the ratio between the intensities of *Xiap* and β -actin, OD.

Statistical analysis

Data are presented as mean $\pm$ S.E.M. for at least six rats per group. Student's *t*-tests were applied wherever necessary, and statistical analysis of differences between groups was performed by one-way ANOVA followed by Tukey's method. Differences were considered as statistically significant when $P < 0.05$.

Results

Assessment of the induced diabetic state: effect of insulin treatment

Diabetes was confirmed in STZ-injected rats by monitoring weight loss and significant increase in blood glucose levels. Compared with control animals, plasma ALT and AST levels were increased by factors of 3 and 2 respectively in the diabetic animals (data not shown). These results are consistent with the STZ model described by others (Barneo *et al.* 1990, Hwang *et al.* 2005, Fernandes *et al.* 2009).

Table 1 shows bw and blood glucose levels in control rats, diabetic rats (SID), diabetic rats treated with insulin (SID+I), and these same three groups treated with DES or TEM. Prior to STZ injection, the bw of diabetic and control rats was similar, and difference was not statistically significant. Thirteen days after STZ administration, the bw of diabetic rats was significantly lower than that of the control group. At this time, the blood glucose levels were significantly increased in SID rats, as compared to control animals. Insulin treatment increased bw and decreased blood glucose levels, reaching the control group values. Treatment with the antioxidants/hydroxyl radical scavengers DES or TEM produced no changes in these parameters.

Assessment of insulin action

In order to assess whether insulin was exerting an anti-apoptotic effect in our experimental model, we evaluated the classical pathway of hormone action PI3K/AKT (Shepherd *et al.* 1998, Virkamaki *et al.* 1999).

We determined the levels of the regulatory subunit of PI3K p85 α and also the levels of P-AKT. AKT has been

Table 1 Assessment of the induced diabetic state. Effect of insulin-treatment. Values are means $\pm$ S.E.M. ($n=6$ animals per group)

	Control	SID	SID+I	SID+DES	SID+DES+I	SID+TEM	SID+TEM+I
Blood glucose (nmol/l)	6.0 $\pm$ 0.5	23.9 $\pm$ 2.2*	8.7 $\pm$ 1.2* [†]	21.1 $\pm$ 4.1*	7.9 $\pm$ 2.2* [†]	25.3 $\pm$ 3.3*	8.1 $\pm$ 2.0* [†]
Body weight (g)	447.3 $\pm$ 16.2	229.0 $\pm$ 31.5*	397.7 $\pm$ 21.6* [†]	266.2 $\pm$ 21.3*	366.5 $\pm$ 22.1* [†]	250.4 $\pm$ 16.3*	347.4 $\pm$ 24.7* [†]

Control, vehicle; SID, streptozotocin-induced diabetes; SID+I, streptozotocin-induced diabetes treated with insulin; DES, desferoxamine; TEM, tempol. * $P < 0.05$ versus control; [†] $P < 0.05$ versus SID.

implicated in the suppression of apoptosis through inactivation of several components of the cell death machinery such as BAD (Datta *et al.* 1997, Galetic *et al.* 1999).

As expected (Nawano *et al.* 1999, Katso *et al.* 2001), the diabetic state reduced significantly both PI3K p85 α

and P-AKT expression, as compared to controls. The treatment with insulin restored the levels of both proteins to normal values, thus evidencing the ability of the hormone to regulate the PI3K/AKT pathway in the liver (Fig. 1A–C).

Figure 1 Assessment of the insulin action. Effect of insulin on (A) regulatory subunit p85 α of phosphoinositol-3 kinase activity (PI3K p85 α), (B) total AKT (AKTt), (C) phosphorylated AKT (P-AKT), and (D) mitochondrial BAD protein expression. The results obtained for all experimental groups are shown as follows: control (C) (white bar), control group of animals injected with sodium citrate vehicle; SID (black bar), streptozotocin (STZ)-induced diabetic rats received an i.p. injection of STZ (60 mg/kg body weight); SID+I (gray bar), on day 15 post STZ treatment, insulin was administered s.c. to SID rats twice a day (at 0800 and 2000 h) for 15 days; SID+DES (black vertically stripped bar), desferoxamine (100 mg/kg body weight, i.p.) was administered to rats, once a day, in saline solution starting 15 days after injection of STZ and for 15 days; SID+DES+I (co-administration) (gray vertically stripped bar); SID+TEM (black horizontally stripped bar), tempol (20 mg/kg body weight, i.v.) was administered to rats, once a day, in saline solution starting 15 days after injection of STZ and for 15 days; SID+TEM+I (co-administration) (gray horizontally stripped bar). Typical examples of western blot of PI3K p85 (A), phospho-AKT (B), total AKT (C), mitochondrial BAD (D), and β -actin or prohibitin for all the proteins are shown in top panel. In (A), (B), (C), and (D) bottom panels, each bar represents the densitometry expressed in percentage considering control as 100%. Values are the mean $\pm$ s.e.m. of six separated animal sets. Total AKT did not show any change in all the studied groups. PI3K p85 and phospho-AKT showed significant diminution in SID, SID+DES, and SID+TEM when compared to control. Insulin treatment restored PI3K p85 levels and increased phospho-AKT in all groups, thus leading to decreased mitochondrial Bad protein expression (* P <0.05 versus C; # P <0.05 versus SID).

Next, we examined BAD levels in mitochondrial fraction. SID rats showed a significant increase of mitochondrial BAD expression. Insulin reduced the levels of this protein, as expected due to its known anti-apoptotic effect (Fig. 1D).

Hydroxyl radical and lipid peroxidation

2,3-DHBA:SA ratio was determined to evaluate $\cdot\text{OH}$ production. Results are presented in Fig. 2A and B. The diabetic state increased $\cdot\text{OH}$ production (56%) compared with the control group ($P<0.05$), and insulin treatment significantly attenuated this increase observed in SID rats ($P<0.05$), thus evidencing its antioxidant capacity. A dramatic decrease in the production of $\cdot\text{OH}$ was found in SID rats when DES or TEM was administered. Co-administration of insulin and DES or TEM to SID rats did not have any additive effect on the reduction of liver $\cdot\text{OH}$ production compared with that obtained after TEM, DES, or insulin administration respectively (Fig. 2A and B).

It has also been described that the $\cdot\text{OH}$ is one of the possible candidates for triggering LPO. We have monitored MDA levels, an LPO product, and a marker of oxidative damage by TBARS assay. MDA is a reflection of the extent of oxidant status and is considered a good marker of oxidative stress (Wen *et al.* 2006).

MDA levels in liver homogenate of SID animals were significantly higher than those of the control group ($P<0.05$; Fig. 2C). Insulin treatment normalized MDA levels in liver homogenate of the SID group ($P<0.05$), suggesting that it is associated with the diabetic state induced by STZ rather than a direct hepatotoxic effect of the drug. This reduction in MDA was not, however, significantly different from the reduction obtained upon administration of DES or TEM alone (Fig. 2C). No changes were observed in these parameters when rats from the control group were treated with the antioxidants/hydroxyl radical scavengers (data not shown).

Figure 2C shows that treatment with TEM produced a larger reduction of LPO in SID than DES. It is known that TEM reduces the formation of $\cdot\text{OH}$ either by scavenging superoxide anions or by reducing the intracellular concentrations of Fe^{2+} , and so we hypothesize that other free radical intermediates, in addition to the $\cdot\text{OH}$, are contributing to the production of LPO observed in SID rats.

*Analysis of the BCL-XL, BAX, and cytochrome *c* proteins*

We examined the expression of BAX protein in liver mitochondrial and cytosolic fractions, and BCL-XL protein in liver mitochondrial fraction, by western blot analysis in all experimental groups. BAX and BCL-XL are members of the BCL-2 family, which plays a major role as regulators of the apoptotic process: while BAX promotes apoptosis, and BCL-XL protects cells from programmed cell death (Tzung *et al.* 1997). Immunoblot analyses followed by quantitative densitometry

from six separate animal sets revealed that mitochondrial BAX protein levels increased by 60% ($P<0.05$), and a consequent diminution of cytosolic BAX levels was observed when compared to the control group (Fig. 3A).

Treatment of SID rats with insulin (SID+I) markedly decreased the mitochondrial protein levels of BAX reaching the control values; however, protein expression of BCL-XL remained increased when compared to the control group ($P<0.05$). BAX:BCL-XL ratio determines cell survival or death after apoptotic stimuli. Figure 3B shows that mitochondrial BAX:BCL-XL ratio was significantly increased in SID rats, indicating that in the diabetic state, the liver is promoted to an apoptotic state. The ability of insulin to decrease the BAX:BCL-XL ratio produces a shift in cell fate towards survival, although the relationship does not reach the control values.

Co-administration of insulin and DES or TEM to SID rats did not have any additive effect on reduction of liver expression of mitochondrial BAX and BCL-XL proteins when compared to that obtained after TEM, DES, or insulin administration respectively (Fig. 3A and B).

Immunoblot analysis of cytosolic cytochrome *c* showed that there was an increase in its release in SID rats when compared to the control group ($P<0.05$), and a consequent diminution of mitochondrial cytochrome *c* levels was observed when compared to the control group. Cytosolic cytochrome *c* protein levels analyzed by quantitative densitometry was increased by 120% ($P<0.05$) in SID rats compared with the control group. The levels of cytosolic cytochrome *c* were decreased by treatment with insulin compared with the SID group, thus observing the consequent increase in mitochondrial cytochrome *c*. The changes produced by treatment with insulin did not reach the values of the control group, remaining significantly increased as compared to the control group ($P<0.05$; Fig. 3C). Administration of DES or TEM also produced a significant attenuation of cytochrome *c* in cytoplasm when compared with SID, and a consequent augmentation of mitochondrial cytochrome *c* levels was observed when compared with the SID group. The changes produced by treatment with antioxidants/hydroxyl radical scavengers did not reach the values of the control group, remaining significantly increased as compared to the control group ($P<0.05$; Fig. 3C). Co-administration of insulin and DES or TEM to SID rats did not have any additive effects on the reduction of cytosolic cytochrome *c* compared with that obtained after TEM, DES, or insulin administration respectively.

Assessment of apoptotic cell death

Caspase-3 activity and TUNEL assays were performed in order to characterize the effect of diabetic state and insulin treatment on induced apoptosis in the liver. Results are presented in Fig. 4. Caspase-3 activity was assayed in liver cytosolic fraction in all experimental groups and is presented in Fig. 4A. There was a significant increase in the caspase-3 activity in SID rats when compared to the control group

Figure 2 Effect of diabetic state and insulin treatment on hydroxyl radical production. (A) Representative chromatograms of samples obtained for each experimental group are depicted. Inset: a representative chromatogram showing the peak of salicylic acid (SA) obtained with u.v. detector (similar peaks were registered in the chromatograms obtained for each of the seven experimental groups). (B) Bars represent the 2,3-DHBA:SA ratio expressed as percent of the control group. Control (C) (white bar), control group of animals injected with sodium citrate vehicle; SID (black bar), streptozotocin (STZ)-induced diabetic rats received an i.p. injection of STZ (60 mg/kg body weight); SID + I (gray bar), on day 15 post STZ treatment, insulin was administered s.c. to SID rats twice a day (at 0800 and 2000 h) during 15 days; SID + DES (black vertically striped bar), desferoxamine (100 mg/kg body weight, i.p.) was administered to rats, once a day, in saline solution starting 15 days after injection of STZ and for 15 days; SID + DES + I (co-administration) (gray vertically striped bar); SID + TEM (black horizontally striped bar), tempol (20 mg/kg body weight, i.v.) was administered to rats, once a day, in saline solution starting 15 days after injection of STZ and for 15 days; SID + TEM + I (co-administration) (gray horizontally striped bar). Data are expressed as means $\pm$ S.E.M. for at least four rats for each experimental group. (C) Lipid peroxidation levels, expressed as nmol of MDA/mg of protein, were determined in liver homogenates of all experimental groups: control, SID, SID + I, SID + DES, SID + DES + I, SID + TEM, and SID + TEM + I. Data are expressed as mean $\pm$ S.E.M. for at least six rats for each experimental group. (* $P < 0.05$ versus C; # $P < 0.05$ versus SID).

Figure 3 Immunoblot analysis of pro-apoptotic and anti-apoptotic proteins in liver subcellular fractions. The results obtained for all experimental groups are shown as follows: lane 1: control (C) (white bar), control group of animals injected with sodium citrate vehicle; lane 2: SID (black bar), streptozotocin (STZ)-induced diabetic rats received an i.p. injection of STZ (60 mg/kg body weight); lane 3: SID+I (gray bar), on day 15 post STZ treatment, insulin was administered s.c. to SID rats twice a day (at 0800 and 2000 h) during 15 days; lane 4: SID+DES (black vertically stripped bar), desferoxamine (100 mg/kg body weight, i.p.) was administered to rats, once a day, in saline solution starting 15 days after injection of STZ and for 15 days; lane 5: SID+DES+I (co-administration) (gray vertically stripped bar); lane 6: SID+TEM (black horizontally stripped bar), tempol (20 mg/kg body weight, i.v.) was administered to rats, once a day, in saline solution starting 15 days after injection of STZ and for 15 days; lane 7: SID+TEM+I (co-administration) (gray horizontally stripped bar). (A) Mitochondrial and cytosolic BAX protein expression. Typical examples of western blots are shown in top panel for each experimental group. The accompanying bars represent the densitometry expressed in percentage from six separate animal sets, considering control as 100%. Data are expressed as means $\pm$ S.E.M. (B) Mitochondrial BCL-XL protein expression. Typical examples of western blots are shown in top panel for each experimental group. The accompanying bars represent mitochondrial BAX:BCL-XL ratio expressed as percent of the control group of the densitometry obtained for BAX and BCL-XL. Data are expressed as means $\pm$ S.E.M. for at least six rats for each experimental group. (C) Mitochondrial and cytosolic cytochrome *c* expression. Typical examples of western blots are shown in top panel for each experimental group. The accompanying bars represent the densitometry expressed in percentage from six separate animal sets, considering control as 100%. Data are expressed as means $\pm$ S.E.M. (D) Mitochondrial and cytosolic prohibitin expression. Typical example of western blot is shown to assess purification in differential centrifugation steps.

($P < 0.05$). The caspase-3 activity was significantly decreased by insulin treatment when compared to SID rats ($P < 0.05$), while no difference was observed when compared to the control group. By contrast, treatment with antioxidants/hydroxyl radical scavengers resulted in a decreased caspase-3 activity although without reaching the levels of the control group ($P < 0.05$). Co-administration of insulin and DES or TEM to SID rats produced a reduction of caspase-3 activity, reaching the control values.

Diabetic state significantly increased the AI when compared to the control group ($P < 0.05$), while treatment with insulin significantly attenuated the increment in this parameter when compared to the SID group ($P < 0.05$), reaching the control values (Fig. 4B). Treatment with antioxidants/hydroxyl radical scavengers resulted in a decreased AI but without reaching the levels of the control group. Co-administration of insulin and DES or TEM to SID rats produced a reduction of AI, reaching the C values ($P < 0.05$ versus SID).

In Fig. 4C, a representative TUNEL assay for control, SID, and SID+I is showed. TUNEL-positive signal is maximal in the SID group and it is clear that after insulin treatment,

there is a significant reduction of TUNEL-positive cells. In hepatic tissue section, the occurrence of apoptosis was confirmed by hematoxylin and eosin staining. Typical features of apoptosis, such as cellular shrinking with cytoplasmic acidophilia, condensation, and margination of the chromatin, are shown in Fig. 4D.

In no case, the careful histological analysis of liver sections stained with hematoxylin-eosin showed inflammatory foci or necrosis.

Analysis of XIAP protein expression and Xiap mRNA levels

As described in the introduction and previously (Nakagami *et al.* 2002, Jiang & Wang 2004), the translocation of BAX protein into mitochondrial membrane is accompanied by cytochrome *c* release from mitochondria to cytosol, which produces a significant increase in caspase-3 activity, leading to cell death by apoptosis. Insulin treatment produced a significant diminution, but without reaching the control values, in mitochondrial BAX protein and cytosolic cytochrome *c*. Interestingly, the activity of caspase-3 and the AI decreased, reaching the control values.

Figure 4 (A) Effect of diabetic state and insulin treatment on caspase-3 activity. The activity of caspase-3 was determined by means of a fluorometric assay. The bars represent activity expressed in percentage, considering control as 100%. Data are expressed as means $\pm$ s.e.m. for at least six rats for each experimental group. Control (C) (white bar), control group of animals injected with sodium citrate vehicle; SID (black bar), streptozotocin (STZ)-induced diabetic rats received an i.p. injection of STZ (60 mg/kg body weight); SID + I (gray bar), on day 15 post STZ treatment, insulin was administered s.c. to SID rats twice a day (at 0800 and 2000 h) during 15 days; SID + DES (black vertically stripped bar), desferoxamine (100 mg/kg body weight, i.p.) was administered to rats, once a day, in saline solution starting 15 days after injection of STZ and for 15 days; SID + DES + I (co-administration) (gray vertically stripped bar); SID + TEM (black horizontally stripped bar), tempol (20 mg/kg body weight, i.v.) was administered to rats, once a day, in saline solution starting 15 days after injection of STZ and for 15 days; SID + TEM + I (co-administration) (gray horizontally stripped bar) (* $P < 0.05$ versus C; # $P < 0.05$ versus SID; † $P < 0.05$ versus SID + DES; ‡ $P < 0.05$ versus SID + TEM). (B) Effect of diabetic state and insulin treatment on liver apoptosis. Apoptotic index (AI) was expressed as percentage of apoptotic cells scored per 10 000 hepatocytes per slide at a magnification of 400 $\times$. The bars represent AI considering control as 100%. Data are expressed as means $\pm$ s.e.m. for at least six rats for each experimental group. (C) TUNEL assay. A representative TUNEL assay is showed which was performed on liver slides taken from the control, SID, SID + I, SID + DES, SID + DES + I, SID + TEM, and SID + TEM + I groups to determine the number of apoptotic cells. (D) Representative photographs of apoptotic and normal cells are shown stained with hematoxylin-eosin for the morphological analysis. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-09-0462>

Extensive data from both *in vitro* and *in vivo* systems have demonstrated that increasing XIAP, a member of the inhibitor family of apoptosis proteins (IAPs), can suppress apoptosis triggered by diverse stimuli (Case *et al.* 1999, Wang *et al.* 2007). XIAP can bind directly to procaspase-9 and activated caspase-3, preventing apoptosis (Roucou *et al.* 2001). To address whether the anti-apoptotic action of insulin on the liver is exerted through XIAP activation, the immunoblot analysis of cytosolic XIAP was performed. Our results show that there was a decrease in the expression of this protein in SID rats when compared to the control group (Fig. 5A, $P<0.05$). Interestingly, insulin significantly increased XIAP protein. Administration of DES or TEM did not produce a significant increase of XIAP protein in cytoplasm when compared to the SID group (Fig. 5A, $P<0.05$). Next, we investigated whether the action of insulin is exerted through mRNA induction. Figure 5B shows a marked diminution of *Xiap* mRNA levels in the SID group, whereas the insulin treatment revealed *Xiap* mRNA induction.

Taken together, these data suggest that in diabetes, hyperglycemia increases the production of $\cdot\text{OH}$ in the liver, leading to the translocation of pro-apoptotic protein Bax from cytoplasm to mitochondria, increasing the release of cytochrome *c* from mitochondria to cytosol. This event leads

to the activation of caspase-3, which coupled with the decline of anti-apoptotic protein XIAP, and conduces to apoptotic cell death. Insulin, through the reduction of hyperglycemia, helps to decrease the production of $\cdot\text{OH}$ radical, which produces a diminution in the translocation of BAX from cytosol to mitochondria and cytochrome *c* release, although not reaching the values of the control group. Interestingly, however, there was a normalization of the activity of caspase-3 and AI. These results may be explained by the induction of anti-apoptotic protein XIAP by insulin; this fact was demonstrated, to our knowledge, for the first time.

Discussion

The results obtained from this study demonstrate that apoptosis occurs in the diabetic liver. Importantly, this study has identified that $\cdot\text{OH}$ contributes partially to mitochondrial cytochrome *c* release and caspase-3 activation, which are associated with hyperglycemia-induced liver apoptosis. Furthermore, our results show that insulin treatment of diabetic rats produces a decrease in hepatic apoptosis, at least in part, by induction of the IAP (XIAP).

Diabetes is known to be a major disorder in which oxidative stress and free radical production have been implicated through several lines of evidence (Hinokio *et al.* 1999, Suzuki *et al.* 1999, Brownlee 2001). ROS have been defined as an autocatalytic mechanism that can lead to programmed cell death (apoptosis; Jones *et al.* 2000). Regulation of cell death by apoptosis may be another determinant of liver structure and lesion formation (Koniaris *et al.* 2003). It has become increasingly clear that the process of cell death by apoptosis is a relatively ubiquitous phenomenon in a variety of cell types, including hepatic cells (Patel *et al.* 1999). The mechanisms regulating this process are complex and incompletely understood.

To study the role of $\cdot\text{OH}$ in LPO and in apoptosis in the liver of STZ-induced diabetes rats, diabetic animals were treated with the potent iron chelator DES (Knecht & Mason 1993) and in another set of experiments, with a direct scavenger of hydroxyl radicals TEM, which has also been reported to reduce the formation of $\cdot\text{OH}$ by scavenging superoxide anions (Chatterjee *et al.* 2000). The strong inhibition elicited by both DES and TEM on LPO and apoptosis clearly establishes a connection between $\cdot\text{OH}$ production and both LPO levels and apoptosis. Our *in vivo* studies demonstrated that hyperglycemia leads to an increase in $\cdot\text{OH}$ production in rat liver, which was significantly reduced by both TEM and DES. Co-administration of both DES/insulin and TEM/insulin did not provide any additional beneficial effects compared to that obtained using either DES or TEM or insulin alone. However, treatment with TEM shows a larger reduction of LPO in SID rats than the one observed in the treatment with DES. It is known that TEM reduces the formation of $\cdot\text{OH}$ either by scavenging

Figure 5 (A) Immunoblot analysis of XIAP expression in liver cytosolic fraction. Lane 1, control (C) (white bar); lane 2, SID (black bar); lane 3, SID+I (gray bar); lane 4, SID+DES (black vertically striped bar); lane 5, SID+DES+I (gray vertically striped bar); lane 6, SID+TEM (black horizontally striped bar); lane 7, SID+TEM+I (gray horizontally striped bar). The accompanying bars represent the densitometry expressed in percentage from six separate animal sets, considering control as 100%. Data are expressed as means $\pm$ S.E.M. (B) Insulin-induced up-regulation of *Xiap* hepatic mRNA. Total mRNA was isolated, and *Xiap* and β -actin mRNAs were assessed by RT-PCR analysis. Relative mRNA levels were quantitated, and *Xiap* levels were normalized to β -actin mRNA, and the ratio is expressed in arbitrary units. Ratios are presented in graphical form considering control as 100% (mean $\pm$ S.E.M.), and the data are representative of four experiments.

superoxide anions or by reducing the intracellular concentrations of Fe^{2+} , which could suggest that other intermediate free radicals than $\cdot\text{OH}$ are also contributing to the production of LPO observed in SID rats.

In the liver, the involvement of reactive oxygen radicals has been suggested in apoptotic cell death of hepatocytes and endothelial cells (Jaeschke 2000). It is well established that members of the Bcl-2 family are critical regulators of apoptosis in a variety of cell types and appear to be cell specific (Gibbons 1995, Evan & Littlewood 1998, Patel *et al.* 1999, Li *et al.* 2005). BAX:BCL-XL ratio determines cell survival or death after apoptotic stimuli. BAX protein has been shown to promote cell death via homodimerization, whereas heterodimerization with BCL-XL results in cell survival (Ronco *et al.* 2002). Our study demonstrates that there is an increased expression of BAX and BCL-XL in the diabetic state. Therefore, while the expression of BCL-XL was also augmented by insulin treatment, pro-apoptotic BAX protein showed a diminution when compared to SID but without reaching the control values. We propose that during the diabetic state there is a relative prevalence of BAX, which promotes cell death by apoptosis. Moreover, we demonstrate that all the treatments (insulin, DES, and/or TEM) produced a significant diminution of BAX:BCL-XL ratio.

It is well established that induction of BAX protein and its translocation from the cytosol to the mitochondria lead to the release of cytochrome *c*, which results in caspase-3 activation inducing apoptotic cell death (Zimmermann *et al.* 2001). Our data show that the up-regulation of BAX may play a key role in the increase of caspase-3 activity by the release of cytochrome *c* from mitochondria, thereby leading to an increase of the AI in the diabetic state. Likewise, these data strongly suggest that insulin, DES, and TEM exert anti-apoptotic actions in the liver through diminution of pro-apoptotic BAX protein (diminution of BAX:BCL-XL ratio). Also, the hormone treatment showed a significant diminution of AI reaching the control value due to a normal caspase-3 activity. Importantly, the present study demonstrated that insulin attenuated hyperglycemia-induced liver apoptosis through decreasing $\cdot\text{OH}$ production.

A large body of evidence has shown the mediation by PI3K and AKT in the anti-apoptotic action of insulin in a variety of cell types (Lawlor & Alessi 2001, Xi *et al.* 2005, Ricci *et al.* 2008). AKT is required to maintain the pro-apoptotic protein BAD inactive (Fernando & Wimalasena 2004). Phosphorylated BAD is sequestered away from the site of action in the mitochondria by binding to cytosolic 14-3-3 proteins (Datta *et al.* 1997, Yano *et al.* 1998).

In our experimental model, we note that the treatment with insulin induces AKT activation and then BAD cytosolic sequestration and decreased levels of mitochondrial Bad (Fig. 1). Our data show that the regulatory subunit p85 α of PI3K exhibits a significant increase in comparison with SID (reaching the control values), which leads to the activation of AKT in agreement with other authors who

suggest that the regulatory subunits can modulate the signals of insulin, i.e. an increase in p85 α leads to an increase in AKT activity (Ueki *et al.* 2003).

In this study, SID rats show mitochondrial cytochrome *c* release, leading to increased activity of caspase-3. As an unexpected finding, insulin treatment of SID does not inhibit mitochondrial release of cytochrome *c* fully, while the activity of caspase-3 shows a complete normalization. One possible hypothesis that could explain this dissociation between cytochrome *c* and caspase-3 activity may be by the action of the IAPs. IAPs are a family of proteins that have been identified as potent cellular caspase inhibitors (Roucou *et al.* 2001). A member of this family is XIAP, which inhibits the caspases that participate in the initiation of the apoptosis cascade (e.g. caspase-9) as well as those that participate in terminal events of the apoptosis cascade (e.g. caspase-3; Holcik & Korneluk 2001). Extensive data from both *in vitro* and *in vivo* systems have demonstrated that increasing XIAP can suppress apoptosis triggered by diverse stimuli (Case *et al.* 1999, Kugler *et al.* 2000). Moreover, Wang *et al.* (2007) have described a diminution of XIAP in muscle of insulin-deficient mice. We checked XIAP as an endogenous protein that would act at the terminal step of apoptosis. Our data show that XIAP is decreased in the liver of insulin-deficient

Figure 6 Proposed mechanism for diabetes enhanced apoptosis in rat liver. STZ-induced hyperglycemia increased BAX more than BCL-XL protein, and also induced translocation from cytosol to mitochondrial membrane leading to cytochrome *c* release and consequent activation of caspase-3 leading to apoptosis. Inhibition of $\cdot\text{OH}$ production by two known inhibitors (desferoxamine or tempol) blocks the translocation of BAX and concomitant cytochrome *c* release. Insulin decreases hyperglycemia, thus leading to an attenuated $\cdot\text{OH}$ production, and consequently attenuates BAX protein mitochondrial levels, leading to the inhibition of caspase-3. Overall, we consider that in STZ-induced diabetes, hyperglycemia, in part via $\cdot\text{OH}$, increases apoptosis through the BAX–caspase pathway. On the other hand, insulin significantly increases XIAP, which is a potent caspase inhibitor, resulting in an inhibition of liver apoptosis.

rat (SID group, Fig. 5). Also, we found that the treatment with insulin increases the XIAP expression levels by inducing its mRNA (Fig. 5).

This is the first study to show that insulin has anti-apoptotic action, at least in part, through XIAP induction in rat liver. Other authors had reported that XIAP is stabilized via the activation of AKT in Kupffer cells (Ashare *et al.* 2007). Moreover, XIAP is known to be phosphorylated at serine 87 by Akt, and this phosphorylation stabilizes the protein (Dan *et al.* 2004). In this sense, our results show increased phosphorylation of AKT in insulin-treated groups, contributing probably to XIAP stabilization.

Taken together, our findings demonstrate that insulin treatment impairs the increase of $\cdot\text{OH}$ production generated by the diabetic state in the rat liver. High levels of this reactive species play an important role in the increase of hepatic LPO observed in the diabetic state, and finally lead to increased pro-apoptotic events by altering the fine balance between the expression of pro-apoptotic and anti-apoptotic proteins. The relative concentration of these proteins is an essential determinant of their final impact on cell fate, and promotion of pro-apoptotic events plays a central role in the development of liver complications in diabetes. Importantly, this study demonstrates that insulin reduced $\cdot\text{OH}$ levels caused by hyperglycemia and exhibited an attenuation of the translocation of BAX to the mitochondria, partially affecting the release of mitochondrial cytochrome *c* to cytoplasm and preventing the activation of caspase-3 producing a slight anti-apoptotic effect. Interestingly, we also show that another way in which insulin exerts its anti-apoptotic action is due to its ability to increase the expression of XIAP protein that appears to be independent of the generation of $\cdot\text{OH}$.

Only the insulin treatment, but not the antioxidants/hydroxyl radical scavenger ones, results in the reversal of the alterations observed in the diabetic state as reduced PI3K, increased mitochondrial BAD, and decreased XIAP. On the other hand, all treatments lead to a reversal of the alterations observed in the diabetic state as increased $\cdot\text{OH}$ production, LPO levels, increased BAX:BCL-XL ratio, increased cytochrome *c* release from the mitochondria, augmentation of cytochrome *c*, and increase of AI.

Overall, we demonstrated that hyperglycemia enhances $\cdot\text{OH}$ levels, induction of BAX protein, and translocation of BAX from cytosol to the mitochondria, leading to the release of cytochrome *c* and consequent activation of caspase-3 with resultant apoptosis. Insulin leads to a decrease of $\cdot\text{OH}$ levels, which produces a slight reduction of BAX:BCL-XL ratio, leading to a mild diminution of cytochrome *c* release from mitochondria to cytosol, and a significant decrease in both caspase-3 activity and AI. Only insulin increases IAP expression (XIAP; Fig. 6).

Taken together, our study demonstrates a partial contribution of $\cdot\text{OH}$ in hyperglycemia-induced liver apoptosis, which may contribute to the disease process in diabetic liver. Also, a new anti-apoptotic signal for insulin is shown, given by an increase in XIAP expression. The control

of hepatic $\cdot\text{OH}$ production and XIAP levels in the diabetic state could be of therapeutic relevance for improvement or delay of the hepatic complications linked to chronic hyperglycemia.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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