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Diabetes and Its Hepatic Complication

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1. Introduction

After food intake, blood glucose levels rise and insulin is released by the pancreas to maintain homeostasis. In the diabetic state, the absence or deficient action of insulin in target tissues is the cause of hyperglycemia and abnormalities in the metabolism of proteins, fats and carbohydrates. In addition, chronic hyperglycemia, characteristic of diabetes, is responsible for organic dysfunction, being eyes, kidneys, nervous system, heart and blood vessels the most important affected organs. Diabetes mellitus (DM) is a heterogeneous dysregulation of carbohydrate metabolism, characterized by chronic hyperglycemia resulting from impaired glucose metabolism and the subsequent increase in blood serum glucose concentration. The pathogenic equation for DM presents a complex interrelation of metabolic, genetic and environmental factors, as well as inflammatory mediators. Among the latter, it is mostly unclear whether they reflect the disease process or are simply signs of systemic or local responses to the disease [1].

DM affects about 26 million individuals in America and at least 250 million people worldwide (World Health Organization), causing about 5% of all deaths. Besides, the number of affected people is expected to duplicate by 2030 unless urgent measures are taken [2, 3]. Every day, 200 children under 14 years are affected by type 1 diabetes, and this number increases by 3 per cent each year, whereas the analogous increment for preschool children reaches 6 per cent [4]. All these data point out the epidemic character of DM.

2. Animal models for the study of diabetes

Rats and mice are animals commonly used for studying the effects of diabetes. Type 2 DM can be induced in animal models through dietary modification such as the administration of



sucrose, fructose, high fat diet and glucose infusion or through genetic manipulation such as db/db mice, ob/ob mice, Goto-Kakizaki rats, Zucker diabetic rats and BHE rats [5].

On the other hand, type 1 diabetes can be replicated in animal models through genetic modifications, i.e. non obese diabetic mice (NOD), which spontaneously develop type 1 diabetes in a manner similar to humans [6]. Other animal models genetically selected are the Bio Breeding rats (BB), in which the pancreatic islets are under the attack of immune T cells, B cells, macrophages and natural killer cells. At approximately 12 weeks of age, these diabetic rats present weight loss, polyuria, polyphagia, hyperglycemia and insulinopenia. As in humans, if these rats are not treated with exogenous insulin, ketoacidosis becomes severe and fatal [7]. Another way to obtain experimental animals with type 1 diabetes is through the administration of chemicals such as alloxan or streptozotocin [8-10]. In our laboratory, we have shown that treatment with streptozotocin causes alterations in biliary excretion during the first seven days post-injection of the drug, becoming normalized 10 days after injection [10, 11]. This is the reason why studies of liver function during streptozotocin-induced diabetic state should be performed fifteen day after injection of the drug. In our work, streptozotocin-induced diabetes (SID) was induced by a single dose of streptozotocin (STZ) (60mg/kg body weight, i.p., in 50 mM citrate buffer, pH 4.5). Control rats were injected with vehicle alone. Fifteen days after STZ injection, a time when the toxic effect of the drug on the liver has disappeared [9, 10], serum glucose levels were tested by means of the glucose oxidase method (Wiener Lab., Rosario, Argentina) in samples obtained from diabetic and control animals. Successful induction of diabetes was defined as a blood glucose level of > 13.2 mmol/l. Between 10 and 12 A.M. the rats were weighed, anesthetized with sodium pentobarbital solution (50 mg/kg body weight, i.p.) and euthanatized. Blood was obtained by cardiac puncture and plasma was separated by centrifugation. Livers were promptly removed and hepatic tissue was either processed for immunohistochemical studies or frozen in liquid nitrogen and stored at -70 °C until analytical assays were performed.

3. Diabetes and inflammation

Inflammation represents a protective response to the control of infections and promotes tissues repair, but it can also contribute to local tissue damage in a broad spectrum of inflammatory disorders. The inflammatory responses are associated with variations of a wide array of plasma proteins and pro-inflammatory cytokines. The acute-phase response is a systemic reaction in which a number of changes in plasma protein concentrations, termed acute-phase proteins, may increase or decrease in response to inflammation [12]. Modifications in the plasma concentration of acute-phase proteins are largely dependent on their biosynthesis in the liver and changes in their production are influenced by the effect of proinflammatory cytokines such as IL-1, IL-6 and tumor necrosis factor alpha (TNF- α) on the hepatocytes. These cytokines are produced during the inflammatory process and they are the main stimulators of acute-phase proteins and other markers of chronic inflammation commonly detected in cardiovascular diseases, diabetes mellitus, osteoarthritis, and rheumatoid arthritis [13, 14].

Chronic hyperglycemia can directly promote an inflammatory state where the increase in cytokines can lead to destruction of the pancreatic beta cells and dysfunction of the endocrine pancreas in diabetes type 1 and 2. [15]. There is evidence that autocrine insulin exerts protective anti-apoptotic effects on beta cells and that it inhibits the suppressor of cytokine signaling (SOCS), which is induced by various cytokines and lead to apoptosis of the beta cell [16]. Commonly, DM type 1 and type 2 are considered inflammatory processes [17, 18] as there is a significant increase in interleukin (IL) IL-6, IL-18, IL-1 and TNF- α in blood of patients with this disease [19, 20].

Futhermore, chemokines (ligands 2 and 5 chemokines CCL2, CCL5 and CX3CL1), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule -1 (VCAM-1) and nuclear transcription factor κ B (NF κ B) are involved in the development and progression of the disease [21, 22]. In this connection, we have demonstrated that hyperglycemia increases the production of hydroxyl radical in the liver of streptozotocin-induced diabetic rats [23]. In addition, the increase in oxidative stress induced by hyperglycemia and inflammation conduces to development of associated diseases such as diabetic nephropathy [17, 21].

The role for pro-inflammatory cytokines in regulating insulin action and glucose homeostasis and their function in type 2 diabetes has been suggested by several lines of evidence. High TNF- α levels are related to the pathophysiology of insulin resistance and type 2 diabetes [24]. The mechanisms that govern the association between the increased synthesis of inflammatory factors and type 2 diabetes are still being elucidated. In macrophages, adipocytes, antigen-presenting B-cells, dendritic cells, and Kupffer cells in the liver, a number of germline-encoded pattern recognition receptors (PRRs), such as the toll-like receptors (TLR), are activated upon ligand binding with conserved structural motifs that are either specific patterns of microbial components (eg, bacterial lipopolysaccharide [LPS]) or nutritional factors (eg, free fatty acids [FFAs]) [25]. Binding to PRRs gives rise to inflammatory responses by mediating downstream transcriptional events that activate nuclear factor-kB (NFkB) and activator protein-1 (AP-1) and their pathways [26]. Upon activation, these intracytoplasmic molecular cascades up-regulate the transcription of pro-inflammatory cytokine genes and, consequently, the synthesis of acute-phase inflammatory mediators and activation of c-Jun N-terminal kinase (JNK) and inhibitor of NFκB kinase-β (IKK). In liver and adipose tissue, these two molecules can inactivate the first target of the insulin receptor (INSR), IRS-1, thereby reducing downstream signaling towards metabolic outcomes [27]. Recent data have revealed that the plasma concentration of inflammatory mediators, such as tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6), is increased in the insulin resistant states of obesity and type 2 diabetes, raising questions about the mechanisms underlying inflammation in these two conditions. Increased concentrations of TNF- α and IL-6, associated with obesity and type 2 diabetes, might interfere with insulin action by suppressing insulin signal transduction. This might interfere with the anti-inflammatory effect of insulin, which in turn might promote inflammation [13].

4. Nitric oxide in TNF- α pathways and apoptosis

As stated above, one of the main cytokines released in these inflammatory processes is TNF- α , which can activate signaling pathways associated with cell survival, apoptosis, inflammatory response and cell differentiation. The induction of the responses mediated by TNF- α occurs through the binding of the cytokine to the receptors TNF-R1 and TNF-R2. Both receptors may mediate cell death, however, TNF-R1 has a death domain while TNF-R2 does not, but it would enhance the cytotoxic effects of TNF-R1. TNF- α is produced primarily by cells of the immune system, such as macrophages and lymphocytes in response to inflammation and infection [28, 29]. The binding of TNF- α to TNF-R1 can promote the activation of NF κ B or initiate the activation of caspases, which play a major role in the execution of programmed cell death or apoptosis (Figure 1) [30]. NFkB stimulates the expression of genes encoding cytokines (e.g. TNF- α , IL-1, IL-6, IL-2, IL-12, INF- γ and CM-CSF), cell adhesion molecules (CAMs), chemokine receptors and inducible enzymes (e.g., COX-2, iNOS). It also increases the expression of molecules involved in regulating cell proliferation, apoptosis and cell cycle progression, such as the cellular inhibitor of apoptosis protein 1 and 2 (c-IAP1 and c-IAP2), TNF-receptor-associated factor 1 and 2 (TRAF-1 and TRAF-2), B-cell lymphocyte/ leukemia-2 (Bcl-2), Fas, c-myc and cyclin D1 [31, 32]. It was found that high levels of glucose can cause apoptosis, in part, through activation of NFkB [33]. Other authors have shown that high glucose levels activate protein kinase C (PKC) pathway and reactive oxygen species (ROS) [34-36]. Furthermore, cytokines and bacterial pathogens can activate iNOS and generate large concentrations of NO, through activation of nuclear transcription factors [37].

4.1. Hepatic expression of TNF-α and TNF-R1, NFκB activity and iNOS expression

We analyzed the hepatic levels of TNF- α and its receptor TNF-R1 by western blot. As shown in Figure 2 (A and B), hepatic levels of TNF- α and TNF-R1 of the diabetic group were higher than those of the control animals (120 % and 300 %, respectively).

We performed inhibition studies of NO production using a preferential inhibitor of iNOS enzyme, aminoguanidine (AG). Fifteen days after the onset of diabetes, a group of rats was separated into different groups and received injections of AG. The groups were as follows: Control group, injected with the vehicle citrate buffer only, and receiving AG in isotonic saline i.p. (100 mg/kg body weight) once a day, beginning 3 days before euthanized (Control +AG) [38], Diabetic group receiving AG i.p. (100 mg/kg body weight) once a day, beginning 3 days before euthanized (SID+AG). The whole study lasted one month. Six animals from each group (Control+AG and SID+AG) were euthanatized and the samples were promptly processed. We examined the expression of iNOS in liver cytosolic fraction by western blot in all experimental groups. Immunoblot analysis followed by quantitative densitometry from six separate animal sets revealed that iNOS increased by 500% (p<0.05) in SID rats compared to the control group (Figure 2 D). Treatment of SID rats with AG markedly decreased the cytosolic protein levels of iNOS, thus reaching the control value.

We also determined the role of TNF- α using ENBREL® (etanercept), a dimeric fusion protein that binds to TNF- α and decreases its role in disorders mediated by excess of TNF- α .

Etanercept mimics the inhibitory effects of naturally occurring soluble TNF- α receptors but has a greatly extended half-life in the bloodstream, and therefore a more profound and long-lasting biologic effect than a naturally occurring soluble TNF-R1 [39]. Etanercept was administered to 6 rats from each group (Control-a-TNF- α and SID-a-TNF- α) in a dose of 8 mg/Kg bw/day twice a week for 15 days.



Figure 1. Schematic mechanisms of NF κ B activation induced by TNF- α signaling pathways.

Administration of etanercept or AG also produced a significant attenuation of both TNF- α and TNF-R1 when compared to SID, reaching the control values (Figures 2A and 2B). Also, in Figure 2 C we show that the increase of TNF- α levels in the liver of streptozotocin-induced diabetic rats leads to a marked up-regulation of the NF κ B pathway. The high levels of

TNF- α due to blood glucose levels increased iNOS expression leading to a high production of NO (see Figure 2 D). Similar findings have been reported in different tissues by other authors [40, 41]. Moreover, we observed that the treatment with etanercept, which blocks TNF- α , leads to a decrease in the expression of iNOS which is increased in the diabetic state. It has been shown that high concentrations of glucose cause an increase in the expression of iNOS induced by cytokines [42] in rat tissues. Consistently, high glucose concentrations do not increase iNOS in the absence of TNF- α [43]. The inhibition of iNOS with a selective inhibitor such as aminoguanidine, also reduced the production of TNF- α , thus evidencing an interaction between TNF- α pathway and the activity of iNOS.



Figure 2. Hepatic TNF- α (Panel A) and TNF-R1 expression (Panel B), NF κ B activity (Panel C) and iNOS expression (Panel D). The results obtained for all experimental groups are shown as follows: Lane 1: Control Control group of animals injected with sodium citrate vehicle; Lane 2: Control+a-TNF- α : Etanercept (8 mg/ kg body weight, i.p.) was administered once a day, twice a week, in saline solution starting 15 days after injection of sodium citrate vehicle and for 15 days; Lane 3: Control+AG: Aminoguanidine (100 mg/ kg body weight, i.p.), was administered once a day, in saline sol-

ution starting 15 days after injection of sodium citrate vehicle and for 3 days before euthanasia; Lane 4: SID: Streptozotocin (STZ)-Induced Diabetic rats received an i.p. injection of STZ 60 mg/kg body weight; Lane 5: SID+a-TNF- α : Etanercept (8 mg/ kg body weight, i.p.) was administered once a day, twice a week, in saline solution starting 15 days after injection of STZ and for 15 days Lane 6: SID+AG: Aminoguanidine (100 mg/ kg body weight, i.p.) was administered, once a day, in saline solution starting 15 days after injection of STZ and for 3 days before euthanasia. Immunoblot analysis of TNF- α (Panel A) and TNF-R1 (Panel B) in total liver lysate. Typical examples of Western blots are shown in top panel for each experimental group. The accompanying bars represent the densitometric analysis of the blots as percentage change from six separate animal sets, expressed as arbitrary units considering control as 100%. Data are expressed as means ± SE. Panel C: NFkB activity is showed as follow: Lane 1: Control; Lane 2: Control+a-TNF- α ; Lane 3: Control+AG; Lane 4: SID; Lane 5: SID+a-TNF- α ; Lane 6: SID+AG.*p<0.05 vs Control; †p<0.05 vs SID. Panel D: Immunoblot analysis of iNOS expression in total liver lysate. Effect of the treatments on each experimental group described above. Typical examples of Western blots are shown in top panel for each experimental group. The accompanying bars represent the densitometric analysis of the blots expressed as percentage change from six separate animal sets. Data are expressed as means ± SE. *p<0.05 vs Control; †p<0.05 vs SID.

4.2. Apoptosis induced by TNF- α

Binding of death receptors to their ligands results in the formation of an intracellular death domain (DISC: death-inducing signal complex) generated by the recruitment of two molecules: the death domain-associated TNF-R1 (TRADD: Tumor necrosis factor receptor type 1-associated death domain protein) and protein-associated death domain Fas (FADD: Fas-Associated protein with Death Domain). This complex also recruits procaspase-8 protein, which is activated by proteolysis. Releasing of the active fragment caspase-8 induces the activation of other caspases in the cytosol [44]. Thus, the DISC complex is responsible for the activation of the caspase cascade leading to apoptosis and/or activation of the kinase signaling pathway involved in apoptosis and JNK (c-Jun N- terminal kinase), resulting in the expression of genes through NFkB or AP-1 (activator protein-1) [45, 46]. The activation of caspase-8 that was induced by activation of the death receptor is followed by excision of Bid protein generating an active fragment of 15 kDa, truncated Bid (Bid-t). Bid-t protein is translocated into the mitochondria and interacts with other proteins of the Bcl2 family (Bax and Bak) and induces the release of apoptogenic factors such as SMAC (second mitochondria-derived activator of caspases) / DIABLO (direct IAP protein with low pI), AIF (apoptosis inducing factor) and the release of cytochrome c into the cytosol forming the apoptosome complex with APAF-1 and procaspase-9. In the apoptosome, procaspase-9 is proteolysed to its mature form, which then activates effector caspase-3, ultimately leading to apoptosis [47, 48].

We performed studies in STZ-induced diabetic rats of both, expression of activated caspase-8 and its activity in liver cytosolic fraction. We observed a substantial increase in activated caspase-8 in the diabetic state (Figure 3 A). Administration of etanercept or AG showed a reduction of both activated caspase-8 expression and its activity as compared to STZ-diabetic rats. We also examined the expression of t-Bid in cytosolic fraction and in liver mitochondrial fraction by western blot in all experimental groups. Immunoblot analysis followed by quantitative densitometry revealed that mitochondrial t-Bid protein levels increased by approximately 50% (p<0.05) in STZ-diabetic rats when compared to the control group (Figure 3 B). Administration of etanercept or AG produced a significant attenuation of Bid-t in the mitochondrial fraction when compared to SID. According to that described by other authors in different tissues [49, 50] the anti–TNF- α (etanercept) treatment was demonstrated to produce a declination in the response of receptor TNF-R1 to TNF- α (diminished activated caspase-8 expression and activity and mitochondrial protein t-Bid, as compared to SID group). Treatment with the iNOS-inhibitor showed a significant decrease of activated caspase-8 expression and activity when compared to STZ-induced diabetic rats (Figure 3 A). Also, we evaluated the activation of c-Jun N-terminal kinase (JNK), a member of the family of the mitogen-activated protein kinases (MAPK). The administration of both etanercept and AG prevents the hyperglycemia-induced phosphorylation of JNK (Figure 3 C).



Figure 3. Panel A: Activated Caspase-8 expression and activity in diabetic liver: Protein immunoblot analysis and fluorometric assessment of activity of casapase-8 were performed in cytosolic fraction. Activities represented as bars are shown in arbitrary units. Data are expressed as means ± SE for at least six rats per experimental group. **Panel B:** Immunoblotting of cytosolic BID and t-BID expression in mitochondria-enriched fractions of diabetic liver and effect of different treatments in experimental groups as was described in Figure 2. Typical examples of Western blots are shown

for cytosolic BID and mitochondrial t-BID in top panel for each experimental group. The accompanying bars represent the densitometric analysis of the blots for t-BID expressed as percentage change from six separate animal sets. Data are expressed as mean \pm S.E. *p<0.05 vs Control; †p<0.05 vs SID. **Panel C:** Western blot analysis of p-JNK in the liver tissue of diabetic animals and effect of different treatments. Typical examples of Western blots are shown in: Lane 1: Control; Lane 2: Control+a-TNF- α , Lane 3: Control+AG, Lane 4: SID, Lane 5: SID+a-TNF- α , Lane 6: SID+AG. The accompanying bars represent the densitometric analysis of the blots expressed as percentage from six separate animal sets. Data are expressed as means \pm SE. *p<0.05 vs Control; †p<0.05 vs SID.



Figure 4. Panel A: Caspase-3 activity in diabetic rats and effect of the different treatments: Caspase-3 activity was fluorometrically determined. The bars represent activity expressed as arbitrary units. Data are expressed as means \pm SE for at least six rats per experimental group. *p<0.05 vs Control; †p<0.05 vs SID. **Panel B:** Effect of NO and TNF- α on liver apoptosis of diabetics rats: Bars of apoptotic index (AI) represent the percentage of apoptotic cells scored at least 1000 hepatocytes per field in 10 fields of tissue sections at a magnification of 400X. Data are expressed as means \pm SE for at least six rats per experimental group. *p<0.05 vs Control; †p<0.05 vs SID. **Panel C:** TUNEL assay: A representative TUNEL assay was performed on liver slides from the Control group, Control+a-TNF- α , Control+AG, SID, SID+a-TNF- α and SID+AG groups.

An early study had demonstrated that the activation of JNK is associated with increased TNF-induced apoptosis in hepatocytes [51]. In this connection, our results demonstrate that diabetes leads to the activation of JNK, inducing an increase of the apoptotic index. Moreover, we demonstrated that the decrease of TNF- α levels by etanercept treatment seems to completely abolish the observed activation of JNK induced by the diabetic state, thus leading to a decrease of apoptosis (Figures 3 and 4). We assessed apoptotic cell death by determining caspase-3 activity and performing TUNEL assays. There was a significant increase in caspase-3 activity in SID rats when compared to the control group (p<0.05). The administration of etanercept or AG to SID rats significantly decreases caspase-3 activity as compared to SID rats (p<0.05) (Figure 4 A). Figure 4 B shows Apoptotic Index (AI) expressed as a percentage. Apoptotic cells were identified in all experimental groups. Typical features of apoptosis, such as cellular shrinking with cytoplasmatic acidophilia, condensation and margination of chromatin were corroborated by hematoxylin-eosin staining. The diabetic state significantly increased the AI when compared to the control group (p<0.05), while treatments with etanercept or AG significantly attenuated this increment when compared to SID group (p<0.05), even reaching the control values (Figure 4 B). In Figure 4 C a representative TU-NEL assay for Control, SID, SID+etanercept and SID+AG is showed. TUNEL-positive signal is maximal in the SID group and it is clear that after the different treatments there is a significant reduction of TUNEL-positive cells.

Our results clearly show that in the liver of STZ-induced diabetic rats there is an enlargement of caspase-3 activity with the consequent increase in the AI.

5. Diabetes, inflammation and liver apoptosis

Several studies have shown that TNF- α may be involved in viral hepatitis, alcoholic hepatitis, ischemia/reperfusion liver injury, and fulminant hepatic failure. In human disease, serum levels of TNF- α and hepatic TNF-receptors are frequently increased [52]. A research paper recently published by our group demonstrates that the diabetic state induces an increase of TNF- α and its receptor TNF-R1 in the liver [43]. Data presented in this work show that the increase of TNF- α levels in the liver of streptozotocin-induced diabetic rats leads to a marked up-regulation of the NF κ B pathway. NF κ B is one of the key transcription factors involved in triggering the cascade of events that allow inflammation and different research groups have demonstrated its activation in the diabetic liver [53, 54]. The expression of iNOS is closely related to stimulation of NF κ B, whose recognition sites have been identified in the promoter region of the gene encoding for iNOS.

In the liver of diabetic rats we found an increase of TNF- α due to increased expression of iNOS which led to a high production of NO [43]. Similar results have been reported in different tissues by other authors [40, 41]. In our work, we observed that the treatment with etanercept, which blocks TNF- α , leads to a decrease in the expression of iNOS which is increased in the diabetic state. Furthermore, etanercept treatment reduces the production of NO in the liver of streptozotocin-induced diabetic rats. It has been shown that high concen-

trations of glucose cause an increase in the expression of iNOS induced by cytokines [42] in rat tissues. Consistent with this, high glucose concentrations do not increase iNOS in the absence of TNF- α [43]. The inhibition of iNOS with a selective inhibitor such as aminoguanidine also reduced the production of TNF- α , thus demonstrating an interaction between TNF- α pathway and the activity of iNOS.

Figure 5 depicts a summary of the apoptotic mechanisms occurring through TNF- α pathway in the liver in the diabetic state.



Figure 5. Proposed scheme for the mechanism involved in TNF- α - induced apoptosis in liver disease induced by diabetes type 1. In the diabetic state, hepatic TNF- α elevation induces activation of NF κ B, caspase-8 and JNK, thus leading to an increased apoptotic rate.

6. Conclusion

The relevance of the present chapter is to provide further knowledge on the mechanisms underlying the disease process in the liver during an inflammatory process such as type 1 diabetes. The regulation of hepatic TNF- α level and iNOS activity in the diabetic state could be therapeutically relevant for the improvement or delay of the hepatic complications of chronic hyperglycemia.

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