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Chemical and functional changes of human insulin by in vitro incubation with blood from diabetic patients in oxidative stress

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Abstract

Oxidative stress damage to biomolecules has been implicated in several diseases including diabetes mellitus. In the present study, we investigated the effect of oxidative stress in whole blood (WB) from diabetic patients (n = 60) on recombinant human insulin. Insulin was incubated with WB obtained from diabetic patients (DP) who had hyperglycemia (>300 mg/dL) or from 41 healthy volunteers (HV). Whole blood of DP, unlike WB of HV, induced higher values of formazan (142%), dityrosines (279%), and carbonyls (58%) in the insulin residues. Interestingly, the insulin modified by WB of DP showed less hypoglycemic activity in rat (30%) in comparison with insulin incubated with WB of HV. The incubation of insulin in WB from DP induces chemical changes in insulin and a decrease in its biological activity, events that might be associated with the high levels of oxidative stress markers found in the plasma of these patients.

1. Introduction

An imbalance between antioxidant defenses and the production of reactive oxygen (ROS), nitrogen, and chlorine species is widely believed to contribute to the onset of age-related diseases by causing oxidative stress and oxidative damage [1]. Oxidative damage has been implicated in the development of cancer [2], atherosclerosis [3,4], respiratory diseases [5], neurodegenerative diseases (including Alzheimer disease) [6,7], overweight [8], and diabetes mellitus [9].

The continuous generation of ROS by activated leukocytes and platelets during the progression of chronic diseases is conducive to oxidative damage. Oxidative injury includes endothelial disruption, cleavage of lipids, and oxidation of proteins [10,11]. Recent research on diabetes mellitus has focused on oxidative stress induced by ROS [9]. The instability of glucose and free fatty acid levels that occurs with diabetes is associated with the overproduction of mitochondrial ROS. As a result, oxidative stress in the bloodstream increases and significantly contributes to disease mechanisms [12]. Several biochemical pathways associated with hyperglycemia increase ROS generation. These include glucose autooxidation; nonenzymatic protein glycation; mitochondrial ROS overproduction; and the activation of protein kinase C, nitric oxide synthase, xanthine oxidase, aldose reductase, and the polyol pathway. The increase in ROS generation leads to oxidative stress and biomolecular damage that impairs insulin action [13-16].

Several studies have evaluated antioxidant defenses in diabetic patients, examining both how the increase in free radicals induces biomolecular damage and how this damage relates to complications from the disease [9]. Although these

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studies show that ROS plays a role in the pathology of diabetes mellitus, no studies have examined the direct effects of free radicals on insulin in diabetic patients. Some in vivo and in vitro evidence suggests that noninsulin proteins such as hemoglobin and low-density lipoprotein can be impaired by glucose and ROS [17-19]. In addition, studies have demonstrated that ROS modifies insulin in vitro and produces chemical changes that decrease the hormone's biological activity [20,21].

Because ROS production is high in the blood of patients with uncontrolled diabetics and evidence suggests that insulin can be modified by these species, the blood of these patients may chemically and functionally modify the hormone. In this study, we first confirmed the presence of oxidative stress in the blood of patients with uncontrolled diabetes, determining biomarkers of oxidative damage in their plasma. Next, human insulin was incubated in the blood of these patients; and levels of formazan, carbonyl, and dityrosine were analyzed to establish changes in the insulin structure. Finally, using intraperitoneal insulin tests in rats, we determined whether the insulin modifications lead to a decrease in its activity.

2. Materials and methods

2.1. Patients and healthy volunteers

The protocol was approved by the human, animal ethics, and research committees from the Nacional Medical Center "La Raza," Mexican Institute for Social Security; the National Institute of Respiratory Diseases "Ismael Cosio Villegas"; and the School of Medicine of the National Polytechnique Institute. All patients involved signed an informed consent waiver. The trial was conducted in accordance with the ethical principles originating in the Declaration of Helsinki of 1975 as revised in 1983 and was consistent with Good Clinical Practice Guidelines.

The sample size was calculated from $N = 2[(Z\alpha - Z\beta)\sigma]2/\mu 1 - \mu 2$, where the reference parameter was the variance of the malondialdehyde (MDA) in nanomoles per milliliter (Desco et al, 2002), N = number of patient, $Z\alpha = 95\%$, $Z\beta = 80\%$, $\mu 1 = 1.0$ nmol/mL MDA, $\mu 2 = 0.4$ nmol/mL thiobarbituric acid-reacting products, $\sigma = \pm SD = 0.3$, and $n = 2\ [1.96 - (-0.84)0.3]2/1.0 - 0.3$. These calculations resulted in N = 6. However, we assayed samples from 60 patients and 41 healthy volunteers (HV).

A controlled, comparative, experimental study was conducted on 60 type 2 diabetes mellitus patients (DP). Type 2 diabetes mellitus was diagnosed according to the World Health Organization/American Diabetes Association criteria [22]. Inclusion criteria were symptoms and signs related to noncontrolled diabetes mellitus, including glycemia values higher than 300 mg/dL and age higher than 35 years. Noninclusion criteria were as follows: (1) diabetic patients with clinical complications related to the evolution of the disease (nephropathy, retinopathy); (2) smokers or people who had smoked until the past year; (3) the use of antioxidants (vitamin C, vitamin E, α-lipoic acid, β-carotene, probucol, carvedilol, and iron chelators) or prooxidants (primaquine and iron) within the last 3 months; (4) history of coronary heart disease, myocardial infarction, or heart failure (New York Heart Association class III-IV); (5) neurologic diseases (eg, Parkinson disease, multiple sclerosis); and (6) chronic obstructive pulmonary disease.

Forty-one HV were recruited among the staff of the hospital (medical practitioners and nurses). None of the control subjects were diabetic or smokers, on any special diet, taking antioxidants/prooxidants, and/or taking medication related to any chronic disease for at least 6 months before being part of this study.

2.2. Blood samples

Ten milliliters of peripheral venous blood was collected using a heparinized syringe (PISA Mexican Pharmaceutic, Guadalajara, Jalisco, Mexico; 5000 IU/mL). An aliquot of 500 μL was taken for standard biochemical blood measurements, performed with a GEM Premier 3000 automatic analyzer with iQM (Lexington, MA). The remainder of the blood was transferred to an assay tube containing 3.8% sodium citrate solution.

2.3. Plasma biomarkers of oxidative stress

To evaluate the oxidative stress status present in both HV and DP, several biomarkers of oxidative stress were analyzed in the plasma. To measure the levels of lipidic oxidative products and chemically modified proteins resulting from oxidative damage in plasma, 1 mL of blood was obtained from HV or DP. Plasma was obtained by centrifugation at 1400*g* for 15 minutes and used for the following assays: (1) 100 μL of plasma was used to determine circulating lipid damage by measuring thiobarbituric acid-reacting products, such as MDA [23]. Malondialdehyde was measured at 532 nm; and 1,1,3,3-tetramethoxypropane (Sigma-Aldrich, St Louis, MO) was used as standard. (2) The capacity of oxidized proteins to react with nitroblue tetrazolium (NBT), producing formazan, was used as an indirect means of analyzing the degree to which proteins had been modified by oxidative stress. Formazan concentration reflected the hydroxylation of the 3 phenylalanine residues in the B chain of insulin by the hydroxyl radical, a process that generates 3 new tyrosine residues [20,21]. Novel hydroxylation of these tyrosines and other tyrosine residues generated catechol groups (ie, 3, 4 dihydroxyphenylalanine). In the presence of transition metal ions (such as copper or iron), catechol groups generated orthoquinones, which interacted with NBT to generate formazan [24]. The NBT reaction was carried out with 10 μL of plasma, and the absorbance was measured at 530 nm to detect the formazan [24]. The molar extinction coefficient for formazan ($E = 15$ mmol/L^{-1} cm^{-1}) was used to calculate its concentration [24]. (3) Tyrosine dimers (dityrosine) were determined by measuring

fluorescence at excitation and emission wavelengths of 320 and 410 nm, respectively, from a 100-μL aliquot of plasma [25]. The final concentration of dityrosine was calculated from a standard curve constructed using dityrosine synthesized in the laboratory [26]. (4) Free carbonyl groups were measured using 100-μL aliquots of plasma and 1 mL of 10 mmol/L 2,4-diphenylhydrazine (DPNH) [27]. The absorbance was measured at 370 nm to detect the formation of dinitrophenylhydrazones. The molar extinction coefficient for DPNH (E = 22 000/mol/L^{-1} cm^{-1}) was used to calculate the concentration of carbonyl. (5) Total protein was measured as a reference parameter according to Lowry et al [28].

2.4. Determination of insulin oxidation

Recombinant human insulin (Lilly Laboratories México, D.F. Mexico, renamed as *native insulin* for this study) was exposed in vitro to (*a*) an experimental ROS-generating system using the Fenton reaction (control of oxidation) and (*b*) whole blood (WB) from HV or DP. The following experimental procedure was used: 20 IU of native insulin in isotonic saline (10 IU/mL) was introduced into a 7-cm portion of membrane dialysis tubing with a cutoff of 3500 d (Spectrum Laboratories, Rancho Dominguez, CA); this insulin concentration was used considering the minimal amounts of hormone that have to be used to detect the biomarkers values. The dialyzing tubes were incubated at 37°C in (1) 8 mL ROS-generating solution (Fenton reaction) containing 5 mmol/L H_2O_2 and 4 mmol/L $CuSO_4$ for 5 minutes or (2) 8 mL of WB (from HV or DP) for 4 hours. For blood samples, plasma biomarkers were measured at the beginning and at the end of the incubation. Although biomarkers values differences between the blood from HV and DP were observed on shorter incubation times (data not shown), only after 4 hours of incubation were the differences statistically significant. We used 4 hours of incubation on the rest of experiments.

After incubation, the bags containing insulin were dialyzed 3 × 10 minutes against 500 mL distilled water. Five international units (250 μg) of insulin was used to determine the levels of formazan and carbonyls; in the case of dityrosine formation, 0.5 mg (10 IU) of insulin was used. These determinations were performed with the same procedures indicated above.

2.5. Intraperitoneal insulin test

In agreement with the Guidelines for the Care and Use of Laboratory Animals, published by the National Institutes of Health, male Wistar rats weighing 300 ± 5 g were maintained under controlled light-dark conditions at 20°C with food and water ad libitum. They were anesthetized with sodium phenobarbital (50 mg/kg), and native insulin or treated insulin (incubated in Fenton reaction, WB from HV, or WB from DP) was administered intraperitoneally (1.0 IU/kg). Blood samples were taken from the tails at different times (0-50 minutes), and the glucose concentration was measured using a glucose analyzer (Abbott Laboratories, MediSense Products, Bedford, MA). The concentration at time 0 was considered to be 100%, and other glycemia values were normalized accordingly.

2.6. Quantification of plasma iron

It is well known that the presence of redox-active iron can contribute to formation of hydroxyl and cause protein oxidation. To eliminate this possibility, free iron concentration in plasma from HV and DP was quantified before and after insulin incubation experiments. The free iron concentration in plasma was determined by using a colorimetric assay and a Roche/Hitachi 904 chemistry auto analyzer (Renton, WA).

2.7. Statistics

Data are expressed as means ± SD or as percentages. Student t tests and χ^2 tests were used to analyze the clinical characteristics of the study subjects; a paired t test was performed to analyze the oxidative status stress of the blood samples. Analysis of variance (ANOVA) was conducted with a Bonferroni post hoc test and was used to analyze the hypoglycemic effect of insulin incubated with HV and DP blood; we also analyzed the areas under the curve of this effect with a χ^2 test. P values < .05 were considered to be statistically significant. All tests were performed using Prism 5 software (GraphPad, San Diego, CA).

3. Results

3.1. Patients

The clinical characteristics of the study subjects are shown in Table 1. As expected, the glycemia values detected were significantly higher in the DP group (P < .0001); hypoglycemic treatment provided to the diabetic patients is indicated in Table 1.

Table 1
Clinical characteristics of the study subjects

Characteristics	HV	DP	P value
Age (y)	49 ± 10[a]	53 ± 5[a]	NS[b]
Male/female	12/29	22/38	NS[c]
Body mass index (kg/m^2)	25 ± 4[a]	27 ± 6[a]	NS[b]
Blood glucose (mg/dL)	82 ± 10[a]	352 ± 20[a]	<.001[b]
Onset type 2 diabetes mellitus (y)	–	9 ± 2[a]	–
Antidiabetic treatment			
		No. of patients	
Glibenclamide	–	35	–
Metformin	–	4	–
Glibenclamide/metformin	–	10	–
Insulin	–	11	–

Data presented are numbers of patients. n = 41 (control group) and n = 60 (DP group). NS indicates not significant.
[a] Mean ± SD.
[b] Student t test.
[c] χ^2 test.

3.2. Plasma oxidation biomarkers

Table 2A shows the initial concentrations of oxidative stress biomarkers in plasma from HV and DP (0 hour). Data obtained indicated the presence of high levels of oxidation biomarkers related to protein damage in the DP group ($P < .001$). The values obtained for dityrosine formation and total carbonyls were 135% and 50% higher, respectively, in the DP as compared with the HV group (Table 2A). To analyze the effect of oxidative stress present in DP blood on insulin, the hormone was incubated in this patient's blood for 4 hours at 37°C. We then evaluated the levels of oxidation biomarkers in insulin and plasma; HV blood was used as a negative control for oxidative stress ("Material and methods").

The levels of plasma oxidation biomarkers after 4 hours of incubation at 37°C increased in both plasma groups. However, all the increases in the DP group were significantly higher in comparison with values detected in HV plasma ($P < .01$) (Table 2B). In HV plasma, the amounts of MDA, formazan, and total carbonyl increased by 54%, 97%, and 25%, respectively. In DP, levels increased 447%, 389%, and 19%, respectively. Interestingly, the DP plasma samples, unlike the HV plasma, exhibited an increase in the concentration of dityrosines (20%) (Table 2B).

Because the liberation of iron from hemoglobin (hemolysis) could exert an oxidative effect on insulin, we verified the absence of hemolysis in our samples by quantifying levels of free iron in plasma before and after the incubation period. The values obtained for HV were 59.20 ± 34.82 (time 0) and 69.80 ± 37.45 Fe^{2+} μg/dL (4 hours). The DP displayed values of 58.50 ± 29.27 and 64.00 ± 29.58 Fe^{2+} μg/dL at time 0 and 4 hours, respectively. Nevertheless, no differences were detected when intergroup comparisons were performed.

3.3. Biomarkers of insulin oxidation

We observed a significant ($P < .001$) increase of 142% in the formation of formazan when insulin was exposed to WB from DP, in comparison with insulin exposed to WB from HV (160 ± 3.61 vs 66.06 ± 3.40 nmol formazan per milligram protein) (Fig. 1). The amounts of formazan on native insulin and insulin exposed to the Fenton reaction (ROS-generating system) (7.46 ± 1.26 and 25.26 ± 7.02 nmol formazan per milligram protein, respectively) showed lower values than those observed for insulin incubated with blood from HV (Fig. 1). The increases in formazan concentration indicate chemical modifications such as the hydroxylation of phenylalanine, the generation of new tyrosine residues, and the formation of catechol groups and quinones. On the other hand, significantly more dityrosines were formed in insulin after incubation in WB from DP ($P < .001$) as compared with incubation in WB from HV (Fig. 2). The values obtained after incubation were 1.48 ± 0.10 for insulin in WB from DP and 0.39 ± 0.04 pmol/mg protein in HV blood. For native insulin and insulin exposed to the Fenton reaction for 5 minutes, the values were 0.23 ± 0.03 and 1.00 ± 0.15 pmol/mg protein, respectively. These findings indicated the presence of other modifications on insulin, as well as an important role for ROS in the generation of tyrosine dimers. Fig. 3 shows the concentrations of carbonyls in the insulin molecule that were exposed after its incubation with WB. The control group's values were 0.4091 ± 0.1552 for native insulin and 0.54 ± 0.11 nmol osazone per milligram protein for insulin exposed to the Fenton reaction. For insulin exposed to WB from DP, the value obtained was 58% higher ($P < .001$) than that for insulin exposed to WB from HV (8.22 ± 0.38 and 5.2 ± 0.48 nmol osazone per milligram protein, respectively). Nearly 10 times fewer carbonyls were formed in native insulin and insulin exposed to the Fenton reaction as compared with insulin incubated in HV blood. Although these results suggested structural modifications in insulin such as oxidation of amino acidic residues and damage to the secondary structure, it is important to keep in mind the possible insulin modification by adduct formation and glycosylation process.

Table 2
Oxidative status

A

		MDA (μmol/L)	Formazan (nmol/mg protein)	Dityrosines (pmol/mg protein)	Protein carbonyls (nmol osazone/mg protein)
0 h	HV	6.48 ± 0.63	5.17 ± 0.38	126.3 ± 12.6	0.65 ± 0.037
	DP	6.44 ± 0.32	5.56 ± 0.43	296.8 ± 9.56*	0.99 ± 0.023*

Student *t* test was used in the intergroup analysis (HV vs DP) at initial time (0 h).

B

		MDA	Formazan	Dityrosines	Protein carbonyls
HV	0 h	6.48 ± 0.63	5.17 ± 0.38	126.3 ± 12.6	0.65 ± 0.037
	4 h	9.99 ± 0.97†	10.22 ± 0.39†	126.7 ± 8.04	0.81 ± 0.105†
DP	0 h	6.44 ± 0.32	5.56 ± 0.43	296.8 ± 9.56	0.99 ± 0.023
	4 h	35.22 ± 2.87†	27.22 ± 1.79†	355.5 ± 29.2†	1.18 ± 0.71†

Paired *t* test was used to compare HV or DP before and after incubation (0 vs 4 h).

Plasmatic concentration of biomarkers 0 and after 4 hours of in vitro incubation (37°C).

* $P < .001$.

† $P < .01$.

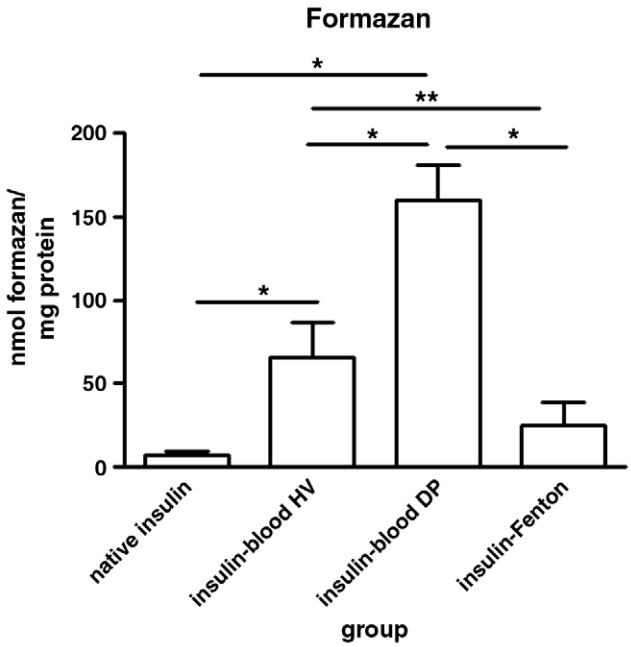

Fig. 1. Formazan production by insulin incubated in blood. Formazan production by insulin incubated in WB of DP was significantly higher (*$P <$ .001) than that in insulin incubated in WB of HV and (**$P <$ .01) Fenton reaction (5 minutes). Data are expressed as means ± SD. Data were analyzed by ANOVA and Bonferroni post hoc test.

3.4. Hypoglycemic effect

The data provided by oxidative markers suggest that structural changes occurred in insulin after its incubation with DP blood; we decided to analyze whether these chemical modifications reduced insulin functionality. Table 3 and Fig. 4 show the glycemia levels and the hypoglycemic effect induced by the insulin modified in vitro (ie, incubated with WB from DP) after intraperitoneal administration in rats. Analyses of the area under the curve and the slopes of insulin-induced effects indicate an inhibition of the biological activity of insulin 50 minutes after it was administered to the rats. This reduction in activity was greater when the hormone was incubated in WB from DP. Similar results were observed when insulin was exposed to the Fenton conditions (Table 3). These results suggest that the modifications generated in insulin are enough to limit the hormone's activity.

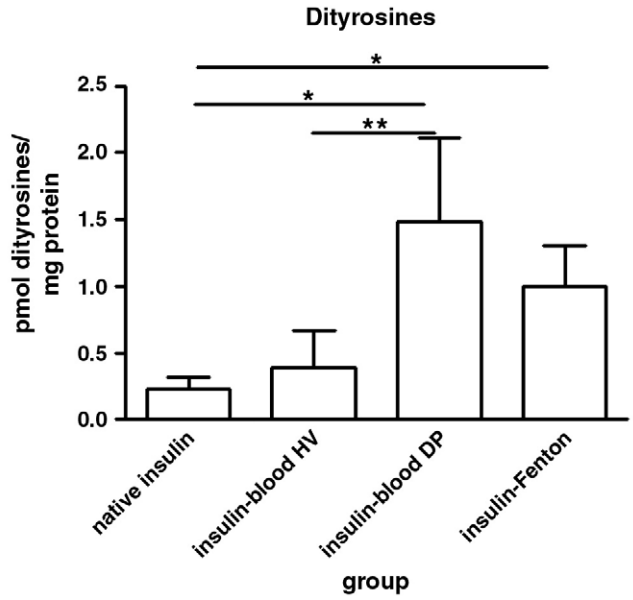

Fig. 2. Dityrosine production by insulin incubated in blood. Higher numbers of tyrosine dimers were formed in insulin incubated in WB of DP or Fenton reaction than in the hormone incubated in WB of HV (*$P <$ .001). Data are expressed as mean ± SD. Data were analyzed by ANOVA and Bonferroni post hoc test.

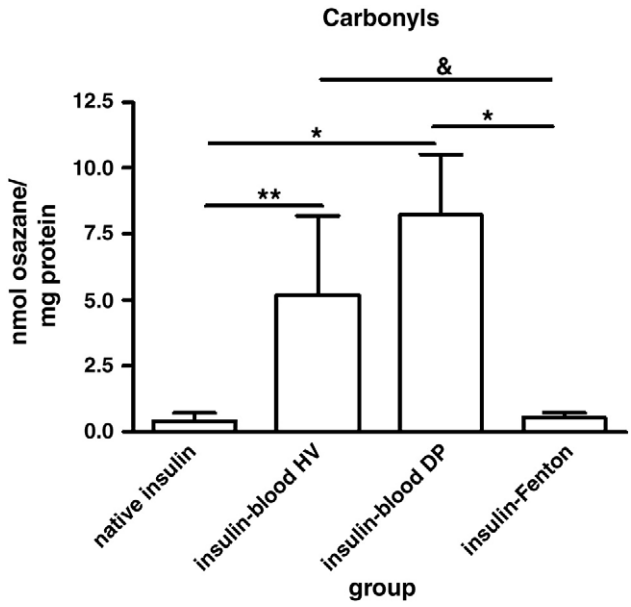

Fig. 3. Carbonyl group exposed by insulin incubated in blood. Higher values of carbonyl groups were generated in the insulin molecule incubated with WB of HV and DP; however, WB of DP had a significantly higher effect than HV (*$P <$ .001). Data are expressed as mean ± SD. Data were analyzed by ANOVA and Bonferroni post hoc test.

4. Discussion

In this work, we have demonstrated that the incubation of insulin in the blood of diabetic patients reduces its biological activity. We suggested that the loss of hormone functionality was generated by the chemical modifications induced in its structure by the adverse milieu present in the blood of DP. It is important to mention that insulin modified by diabetic blood was less efficient than native insulin or insulin incubated with the blood of HV. In fact, the hormone incubated with the Fenton reaction (the positive control) exhibited lower formazan and carbonyl concentrations than the insulin incubated with the blood of DP; this finding suggests that other factors, in addition to ROS, participate in the modification of insulin. However, high levels of dityrosine

Table 3
Hypoglycemic effect of insulin incubated with WB from diabetic patients

Treatment	Glycemia to 0 min (mg/dL)	Glycemia after 50 min (mg/dL)	Hypoglycemic effect compared with initial conditions (%)	Area under the curve (mg/[dL min])	Slope (mg/[dL min])	P value < .05
Saline solution	97.00 ± 2.36	91.67 ± 2.5	5	4682 ± 118	−0.091 ± 0.03	Vs all groups
Native insulin	99.33 ± 3.61	48.17 ± 2.78	51	3514 ± 118	−1.040 ± 0.04	Vs DP or Fenton
Insulin-blood HV	100.8 ± 2.48	51.00 ± 3.09	49	3477 ± 133	−1.006 ± 0.05	Vs DP or Fenton
Insulin-blood DP	99.33 ± 5.08	68.33 ± 5.08	31	4057 ± 165	−0.642 ± 0.05	Vs native or HV
Insulin-Fenton	100.2 ± 6.36	61.33 ± 3.14	39	3837 ± 77	−0.759 ± 0.06	Vs native or HV

Data are expressed as means ± SD, analyzed by ANOVA and Bonferroni t test.

in insulin incubated in diabetic blood or subjected to a Fenton reaction confirmed the modification by free radicals. Furthermore, the slope values calculated from the hypoglycemic effects of these insulins showed a slow decrease in blood glucose; this finding suggests that the modification of insulin by free radicals limited its functionality.

The results showed that oxidized products were present in higher levels in the plasma of DP than in the plasma of HV. The high biomarker levels found in DP could be explained by the elevated production of ROS, low antioxidant defenses [29], and high glycemia values. In addition, when the effect of DP blood on insulin was analyzed, we observed its ability to modify insulin in vitro. Biomarker values in the plasma of HV increased when it was incubated with insulin; however, this increase was negligible when compared with the higher values detected in the blood of DP. The increased concentration of biomarkers in the WB of HV suggests that oxidative stress occurred during the experiment; however, this stress had no effect on insulin functionality. In fact, the biggest changes detected in insulin were found

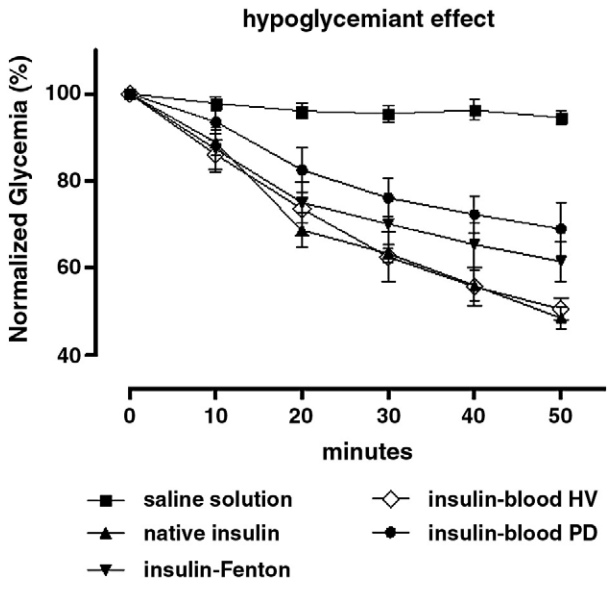

Fig. 4. Hypoglycemic effect of insulin incubated in WB of DP, HV, and Fenton reaction. Insulin incubated in blood of DP showed significantly less biological activity at 50 minutes than insulin incubated in WB of HV and Fenton reaction. Data are expressed as mean ± SD, analyzed by area under and slope.

when the insulin was incubated with diabetic samples; values were significantly higher in these cases than in the samples from HV.

We also suggested that the capacity of blood from diabetic patients to modify insulin resides in the presence of an adverse milieu formed by oxidative stress. This stress is related to chronic disease and includes cellular ROS-generating systems (neutrophils, platelets, and macrophages) [30], the products of oxidative injury, modified proteins, high glucose concentration, and a decrease in the antioxidant system composed of erythrocytes, enzymes, and antioxidant agents [31,32].

It is important to mention that the increase in formazan concentration reflected the hydroxylation of phenylalanine residues (at position 1, 25, 26 in the B chain) in the insulin by hydroxyl radicals, resulting in the generation of new tyrosine residues [20]. Novel hydroxylation of these tyrosines and other tyrosine residues (at positions 14, 19 and 16, 26 in the A and B chains, respectively) generates catechol groups (ie, 3,4-dihydroxyphenylalanine) that, in the presence of transition metal ions (such as copper or iron), generate orthoquinones. Interestingly, some of the modified residues (Phe-B24, 25 and try-B26) in the B chain are part of the subset binding surface, which researchers have suggested is essential to cooperativity in receptor binding [33,34]. The modification of these residues could explain the low insulin activity that was detected. Indeed, this hypothesis is supported by evidence that the alteration of these residues (mutation of phe-B25-Leu and Phe-24-ser) induces insulinopathies [35,36].

Reactive oxygen species also played an important role in the increase in dityrosine formation. The formation of tyrosine dimers requires the oxidation of one electron on L-tyrosine to generate the tyrosyl radical. When 2 tyrosyl radicals react, the major product is *o*-*o*-dityrosine, an intensely fluorescent compound [25]. Higher levels of this compound were detected in insulin after its incubation in blood from DP; this suggests the existence of another mechanism of insulin inactivation. This is possible because the tyrosine residues where these modifications occurred (Tyr-A19, Tyr-B16, and Tyr-B26) are involved in interactions with the receptor [33,34].

Another important piece of evidence pointing to the chemical modification of insulin was the increase in free carbonyl groups after incubation with the blood of DP. The

quantification of carbonyl groups is a marker for oxidative damage in proteins [37]. The exposure of carbonyl groups in the insulin was probably caused by structural modifications such as (1) the oxidation of amino acid residues (such as lysine, arginine, and proline) [38], (2) damage to the peptide bond in the protein backbone [39], (3) damage to hydrogen bonds in the protein's secondary structure, (4) reaction with reducing carbohydrates (glycosylation), and (5) secondary reactions of some lateral chain of lysine residues with lipid peroxidation products (4-hydroxy-2-nonenal and acrolein). This final process leads to the formation of advanced lipoxidation end products (adducts) [40-42] that alter the protein's structure and function and promote the formation of high–molecular-weight protein aggregates. These aggregates, in turn, have been implicated in the development of pathologies via a condition known as *carbonyl stress* [43].

It is well known that the presence of redox-active iron can act as a prooxidant in vitro and can contribute to the formation of hydroxyl radicals. These radicals, in turn, may cause protein oxidation because chemical changes in the insulin molecule can be generated by an increase in iron concentration (ie, hemolysis during the incubation with blood). Free iron in the plasma was measured both before and after incubation to eliminate this possibility. The values obtained demonstrate the absence of an increase in iron concentration and indicate that the chemical modifications of insulin were generated by the adverse milieu in the blood.

In conclusion, we have demonstrated that the incubation of insulin in WB from patients with uncontrolled diabetes induces chemical modifications in the hormone and a decrease in its biological activity. These results point to the existence of an adverse milieu in the blood of patients with uncontrolled diabetes that can modify biomolecules and affect their function. Furthermore, our findings strongly suggest that ROS participates in the development of this adverse milieu. We offer a rationale for further investigation of the involvement of intrinsic mechanisms of oxidative stress-induced molecular injury in diabetes mellitus. We suggest that the inactivation or control of this hostile milieu should be an important part of disease treatment. Such control could be accomplished, for example, by improving the antioxidant capacity of the patient or by inactivating the molecules that are able to induce the formation of adducts such as pyridoxamine [41,44,45].

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