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REACTIVE OXYGEN SPECIES (ROS) INDUCE CHEMICAL AND STRUCTURAL CHANGES ON HUMAN INSULIN *IN VITRO*, INCLUDING ALTERATIONS IN ITS IMMUNOREACTIVITY

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1. ABSTRACT

Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds the endogenous antioxidant defense. Peroxidations induced by ROS are the key of chemical and structural modifications of biomolecules including circulating proteins. To elucidate the effect of ROS on circulating proteins and considering the presence of oxidative stress in Diabetes Mellitus, the effects of ROS, *in vitro,* on human insulin were studied. We utilized the Fenton reaction for free hydroxyl radical ($HO^{\bullet}$) generation in presence of human recombinant insulin measuring chemical changes on its molecular structure. The induced changes in insulin were: a) significant increase on absorbance (280 nm) due to phenylalanine hydroxylation (0.023 +/- 0.007 to 0.13 +/- 0.07). b) Peroxidation products formed on amino acids side branches (peroxyl and alcohoxyl group); measured as increased capacity of reduce nitroblue of tetrazolium (NBT) to formazan (0.007 +/- 0.007 to 0.06 +/- 0.02). c) Increased concentration of free carbonyl groups (8.8 +/- 8.7 to 45.6 +/- 20.2 pmoles dinitrophenylhidrazones/nmol insulin) with lost of secondary structure, and d) Modification of epithopes decreasing the insulin antigen-antibody reactivity measured as a decrease in insulin concentration by RIA. In conclusion, the radical hydroxyl *in vitro* is able to induce molecular modifications on insulin.

2. INTRODUCTION

The reactions of reactive oxygen species (ROS), such as the superoxide anion ($O_2^{\bullet -}$), hydroxyl ($HO^{\bullet}$), and peroxyl free radicals, on biomolecules are important in physiology and pathology (1). Free radicals, usually fragments of normal molecules are entities that contain unpaired electrons. They can be generated within cells as intermediates of normal biochemical pathways, such as those involving redox enzymes and bioenergetic electron transfer. They also may appear under non controlled circunstances, and cause reversible or irreversible damage to macromolecules such as proteins (2), producing chemical alterations and changes on their structure resulting in modifications of biological properties (3) including: inactivation of enzymatic activities (4), and lack of hormonal activity (5), being these molecules in consequence rapidly degraded by intracellular proteases (6).

An important possibility is that oxidized proteins may themselves contain reactive species, which can go on to cause further damage to other proteins and other biomolecules (7). All protein amino acid residues can be attacked or modified by ROS although, Tyr, Phe Trp, His, Met, Cis and the disulphide bound (8) are the preferred targets. The modifications of amino acid residues by $HO^{\bullet}$ and $O_2^{\bullet -}$, and the subsequent changes in their biological

activity, have been used to identify amino acids that are crucial for protein function (9). Protein peroxidations together with modifications of their biological function have been demonstrated in: structural (10) and circulating oxidized proteins, including; IgGs (11), gonadotrophins (5), low-density lipoprotein (LDL) (7). In addition, the albumin (3) can lost its properties as a principal serum antioxidant and became pro-oxidant in the presence of copper, probably by generating ROS. The oxidant effect, and consequently the protein damage, could be neutralized by the presence of antioxidant enzymes or scavengers which generally are antioxidant molecules such as reduced glutathione, thiourea, beta-carotene, alpha-tocopherol, uric acid, ascorbic acid and bilirrubin (12). However in some chronic diseases (13) including diabetes mellitus (14, 15), the production of ROS is higher than the protective antioxidant mechanisms, conducing to oxidative stress (16) and consequently to peroxidation, and modification of proteins (17).

Considering the oxidation susceptibility of; 1) amino acids (Tyr, Phe, Lys, His), in the human insulin primary structure and 2) disulphide bounds and 3) exposition of circulating insulin to ROS during the oxidative stress in the diabetic patient, the purpose of this work was to determine the effects of hydroxyl radicals on the structure of human insulin, in order to demonstrate the characteristics of chemical and structural changes induced *in vitro* by ROS in a milieu similar to the dominant conditions present during insulin circulation under oxidative stress linked to diabetes mellitus (18).

3. MATERIALS AND METHODS

3.1. Reagents

Human recombinant insulin (Humulin*R. Lilly) was obtained from Lilly Lab. México; H_2O_2, $CuSO_4$, trichloracetic acid (TCA), nitroblue tetrazolium (NBT), potasium glycinate, diphenylhydrazine (DNPH), thiourea and glutathione were obtained from Sigma (St. Louis, MO, USA).

3.2. Experimental design

The degree of insulin peroxidation was assessed by three spectrophotometric methods: 1) the absorption changes at 280 nm of insulin, 2) the nitroblue tetrazolium (NBT) reduction (19) and 3) the amount of protein carbonyl groups (20) generated. Additionally radioimmunoassay in solid phase was also carried out in order to assess any immunoreactivity changes.

3.3. Hydroxyl ($HO^{\bullet}$) radical generation

The $HO^{\bullet}$ was produced by the Fenton reaction (5), using the following mixure: 5 mM H_2O_2, 4.0 mM $CuSO_4$, 5 IU of human recombinant insulin equivalent to 33.5 nanomol or 194.55 µg in 1 ml of water. After different times of incubation (0-240 min) at 37 degrees Celsius the reaction was stopped by the addition of 5 % (w/v) TCA and centrifuged at 2000 x*g*. The precipitate obtained was washed twice with 2.5 % (w/v) TCA and centrifugated, and finally resuspended in the solvent utilized for the different assays.

The following groups were included as controls: a) insulin + H_2O, b) insulin + $CuSO_4$, and c) insulin + H_2O_2 in order to be compared with insulin + Fenton reaction.

3.4. Absorption changes (280 nm) and nitroblue tetrazolium (NBT) reduction

The peroxidated and no peroxidated insulin (control) in 1 ml of water were spectrophotometrically analyzed at 280 nm. After, the same volume of NBT 0.28 mM in 2 M potassium glycinate (pH 10) was added to each tube, incubated for 30 minutes and the reduction of NBT to detect formazan was measured at 530 nm (19).

3.5. Protein carbonyl groups

Peroxidated insulin was resuspended in 1 ml of 10 mM 2,4-diphenylhydrazine (DNPH) in 2.5 N HCl. The samples were incubated at laboratory temperature in dark conditions and stirred at 15 min intervals for 60 min. They was precipitated with 20 % (w/v) TCA, centrifuged and washed with 1 ml of 10 % (w/v) TCA and finally washed with ethanol-isopropanol (1:1 v/v) and centrifugated in order to eliminate unreacted DNPH. The pellets were dissolved in 6 M guanidine-HCl, incubated for 10 minutes at 37 degrees Celsius, and the absorbance was measured at 370 nm in order to detect the dinitrophenylhydrazones formation. Molar extinction coefficient for DPNH (E = 22,000/M^{-1} cm^{-1}) was utilized for calculate the carbonyl concentration (20).

3.6. Radioimmunoassay (RIA)

In order to determine if Fenton reaction induced any structural changes in insulin, reflected as changes in immunoreactivity, we measured the concentration of insulin by RIA after the different experimental conditions. Peroxidated insulin for different times (0.5-120 minutes) was compared with similar concentrations of control insulin (3.35 pmol); the commercial kit (Insulin. Diagnostic Products Corporation USA) was utilized. Intra and interassay controls were carried out.

3.7. Statistics

Data were expressed as mean ± SD. Data were analized by ANOVA and Bonferroni *t*-test using the Prism 2.01 software (GraphPad, San Diego, CA, USA); the different groups studied were compared at each incubation time. A P value less than 0.05 was considered statistically significant.

4. RESULTS

4.1. Absorption changes at 280 nm

Figure 1 shows the increase of absorbance (280 nm) of 5 IU of insulin (33.5 nmol or 194.55 microg) in function of exposition time (0-120 minutes) to Fenton reaction ($HO^{\bullet}$ generation). The higher value of absorbance was obtained at 60 minutes of incubation (0.023 ± 0.007 to 1.11 ± 0.206 P<0.001 figure 1A). It was only necessary 3 minutes of insulin exposition to Fenton reaction to obtain statistically significant changes (0.023 ± 0.007 to 0.13 ± 0.07. P<0.05 figure 1B) of absorbance. Mixture insulin with the isolated compounds of the Fenton reaction do not induces changes on absorbance values.

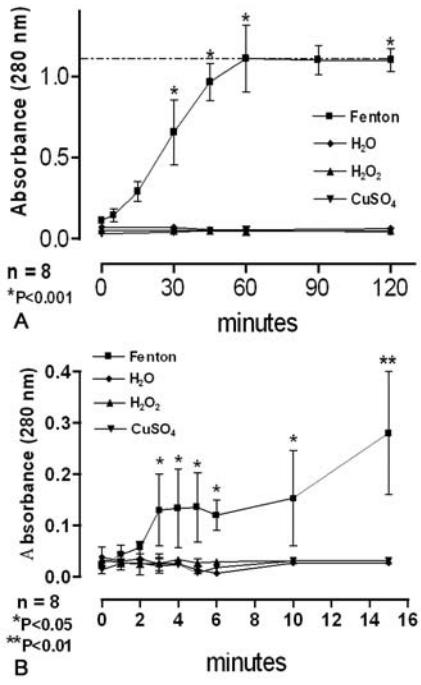

Figure 1. Spectrophotometric increase of insulin absorbance at 280 nm. Absorbance values of recombinant human insulin exposed to HO• at different periods (1A. 0-120 min) (1B 0-15 min). Mean ± SD. ANOVA by each incubation time.

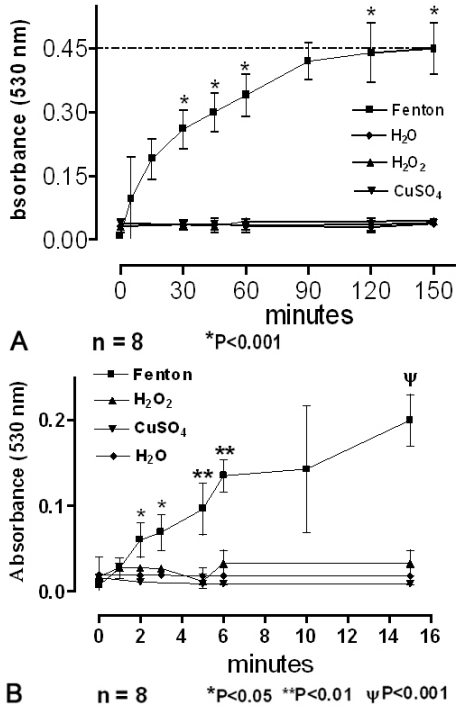

Figure 2. Reduction of nitroblue tetrazolium (NBT). Reaction of the insulin with the NBT in relation with time of oxidation (2A. 0-150 min) (2B. 0-15 min). Mean ± SD. ANOVA by each incubation time.

4.2. Nitroblue tetrazolium (NBT) reduction

The effect of the HO• radical on the insulin and NBT reduction is shown in figure 2. The oxidized insulin during its peroxidation forms intrinsic free radicals (peroxyl and alcohoxyl) that in turn can reduce NBT inducing formazan synthesis. It was obtained the higher absorbance changes at 90 minutes (0.007 ± 0.007 to 0.42 ± 0.044 figure 2A). Significant absorbance changes were obtained within 2 minutes (0.007 ± 0.007 to 0.06 ± 0.02 $P<0.05$ figure 2B). The isolated compounds of Fenton mixture did not induce formazan formation.

4.3. Protein carbonyl groups

Figure 3 shows that oxidized insulin had an increased content of free carbonyl groups measured as a higher capacity to induce the synthesis of dinitrophenylhydrazones (8.8 ± 8.7 to 45.6 ± 20.2 pmol dinitrophenylhidrazones/nmol insulin, figure 3B) during the exposition to Fenton mixture. The 100 % of the reaction was obtained at 120 minutes (8.8 ± 8.7 to 376 ± 108.6 pmol dinitrophenylhidrazones/nmol insulin, figure 3A).

4.4. Radioimmunoassay (RIA)

Figure 4 shows that interaction of HO• with insulin decreases the immunoreactivity of protein. The insulin measured by RIA was reduced 40 % at 30 seconds of reaction (326.8 ± 60 to 199.88 ± 39 micro IU/ml). This effect was time dependent.

5. DISCUSSION

All amino acid side chains of protein are susceptible to an attack by oxidants and free radicals, although some are more vulnerable than others. Thus, exposure of proteins to ROS generating systems may induce changes in their tertiary structure as a consequence of modifications in individual amino acid side chains. Our results showed (figure 1) an increase in spectrophotometric absorbance (280 nm) for oxidized insulin when compared with controls (insulin exposed to the individual components of Fenton reaction). The change of absorbance was evident from 3 minutes (figure 1B) of insulin oxidation. The changes are related with the formation of tyrosine from phenylalanine during hydroxylation processes dependent of the hydroxyl generation from Fenton mixture. The induced effect reached its maximum at 60 minutes of incubation (figure 1A).

Due to hidroxylation of tyrosines forming catechols, and oxido-reduction reactions with copper, there is protein-ortho-quinone formation. Our results show (figure 2) that oxidized insulin can reduce NBT in only 2 minutes (figure 2B) and reached its maximum at 120 minutes (figure 2A). Oxidized insulin might modify other biomolecules and potentially alter its own receptor and/or other circulating molecules or membrane structures. Figure 3 shows the evident increase of phenylhydrazones formed by the phenylhydrazine reaction with exposed carbonyls present in the oxidized insulin. This effect is related to protein tertiary structural changes, as may be the disruption of the

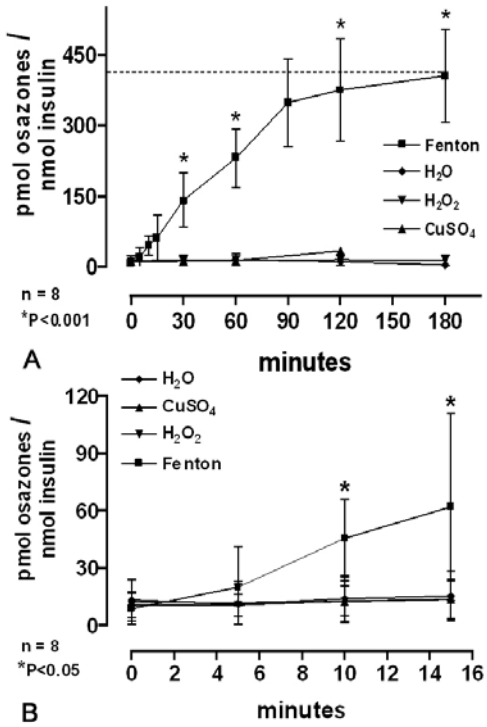

Figure 3. Carbonyl groups exposed. Determination of dinitrophenylhydrazones formation on oxidized insulin (ox-insulin) by the reaction, expressed as pmoles dinitrophenylhydrazones/nmol insulin in function of oxidation time of insulin (3A. 0-180 min) (3B. 0-15 min). Mean ± SD. ANOVA by each incubation time.

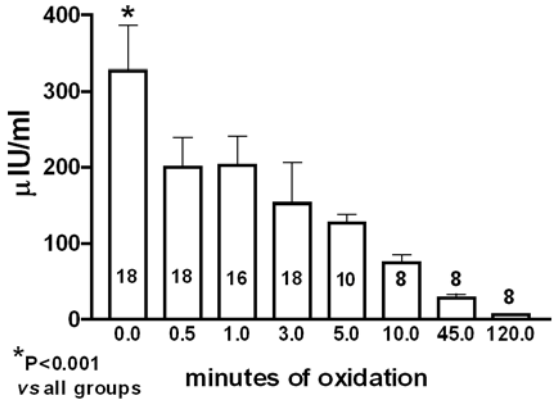

Figure 4. Radioimmunoanalysis. Comparative radioimmunoanalysis assay utilizing oxidized insulin and native recombinant human insulin in solid phase (Coat-A-Counts of Insulin DPC). Intra and Inter assays were carry out. The value of *n* for each group is printed in its respective bar. Mean ± SD. ANOVA.

secondary structure under certain conditions of free radical attack at the alpha carbon of the peptide bond (21), increasing the exposure of carbonyl and the subsequent susceptibility for this chemical reaction (22). An increased concentration of free carbonyls in plasma is considered as indicative of the named *carbonyl stress* (23) that included the exposed free carbonyls from total plasma proteins (albumin, lipoproteins) (24) and now insulin, and several aldehydes as malondialdehyde and acrolein (25) generated by lipoperoxidation process induced by ROS. The carbonyl stress might be included as part of the processes present during oxidative stress, considering that carbonyl compounds, are naturally produced and increased under oxidative stress, representing one of the important biomarkers of protein and tissue damage (23). Our results show that insulin, exposed to individual components of Fenton reaction (controls), has few carbonyl groups available for this reaction as shown in figure 3. The onset of phenylhydrazones synthesis from oxidized insulin was found within 10 minutes and reaches its maximum after 120 minutes of reaction. These results are evidence of the induced insulin structural changes.

Those chemical and structural changes including hydroxylation and increase of free carbonyl groups exposition, affect the identity of amino acid residues and consequently the primary structure of insulin, include the change of antigenic epitopes. This structural modification can explain the decrease on recognition of oxidized insulin in the radioimmunoanalysis assay (figure 4) however, other possibility is that oxidized insulin modifies the antibody by the same way like to NBT, we do not assay this possibilities.

According with data showed here, it is possible to consider the possibility that the assays of radioimmunoassay utilized for insulin determination in plasma samples from patients with diabetes mellitus under oxidative stress, misread the real concentration of circulating insulin. Since plasma glucose, increases significantly in diabetic subjects, hyperglycemia may play and important role in the generation of oxidative stress (26). Similarly, hyperinsulinemia (27) and lipid alterations have been included as contributory factors to oxidative stress generation in diabetes. Oxidative stress has been known to play an important role in the development and progression of nephropathy (28), retinopathy and other diabetic complications (29).

Demonstration of the effect of hydroxyl radical, the most reactive ROS, on the insulin structure might be considered logical, after the numerous reports of modifications on enzymatic systems and the hormone gonadotrophin by ROS. This work describes chemical and structural changes induced by ROS on recombinant human insulin, conducing to changes on insulin antigenic epitopes, leading to a decrease in its immunoreactivity with the antibodies utilized in radioimmunoanalysis. The possibility of altered physiological processes, by injury on circulating hormones including insulin, antibodies, enzymes, coagulation factors and lipoproteins among other vital molecules by ROS produced by different sources, including cigarette smoke and other air pollutants, is currently under study.

In conclusion, insulin can be damaged by $HO^{\bullet}$, and in this condition it can in turn modify other molecules. Our results suggests that the oxidative stress found in the patients with diabetes mellitus (30) is or can be accompanied by a decrease in insulin biological activity, however more work is necessary in order to prove it.

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