

OXIDATION BY REACTIVE OXYGEN SPECIES (ROS) ALTERS THE STRUCTURE OF HUMAN INSULIN AND DECREASES THE INSULIN-DEPENDENT D-GLUCOSE-C¹⁴ UTILIZATION BY HUMAN ADIPOSE TISSUE

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1. ABSTRACT

The formation of dityrosine of human insulin oxidized by metal-catalyzed oxidation system (H₂O₂/Cu) was estimated by fluorescent methods. The oxidation of tyrosine and phenylalanine residues present on the insulin molecule was evident after 2 minutes of *in vitro* oxidation due to the formation of protein-bound dityrosine. The success of oxidative protein modification was followed until available aromatic residues were consumed (60 minutes), measured by their emission at 405 nm. The structural and chemical changes on insulin molecule are related to the loss of biological activity as assessed by measuring the increase of U-¹⁴C-glucose utilization by human adipose tissue in a radiorespirometry system. The oxidation of glucose (¹⁴CO₂ production) of the adipose cells was increased 35 % (301 +/- 119 to 407 +/- 182 cpm/mg in dry weight. P < 0.05) in presence of 0.1 IU and 69 % (301 +/- 119 to 510 +/- 266 cpm/dry weight. P < 0.05) for 1.0 IU of insulin. The recombinant human insulin oxidized for 5 minutes only increased the glucose oxidation by 25 %. In conclusion, these observations show that dityrosine formation and other oxidative chemical changes of insulin due to its *in vitro* oxidation decrease and can abolish its biological activity.

2. INTRODUCTION

Proteins in living entities are subject to continuous turnover as well as biochemical changes, by different kinase and phosphatase systems (1) resulting in multi-functional capacities. These biochemical modifications might change structural properties and biological function due to the continuous interaction with physical and chemical factors including free radicals (2) and other reactive oxygen or nitrogen/chlorine species. Such changes might contribute to the onset and/or development of several chronic diseases through oxidative stress and oxidant damage (3). These processes have been implicated in cancer (4), atherosclerosis (5), degenerative diseases and diabetes.

Oxidative stress results from imbalance between the production of reactive species and antioxidative events. Sies (6) defined it as a disturbance in the pro-oxidant/antioxidant balance in favor of the former, potentially leading to tissue damage. The damage to proteins by reactive species during oxidative stress (7) may be important *in vivo* since it can affect the function of receptors, enzymes, antibodies (8) transport proteins (9) and hormones (10). Recently, we demonstrated that *in vitro*

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exposure of insulin to reactive oxygen species (ROS), induced chemical and structural changes leading to an increased concentration of: 1) free carbonyl groups, 2) peroxidation products, formed on amino acids side branches (peroxyl and alcoxyl group) and 3) phenylalanine hydroxylation producing tyrosine, affecting insulin immunoreactivity (11). Considering the oxidation susceptibility of human insulin, *in vitro* as well as *in vivo*, during circulation due to the presence of ROS generating systems, particularly in diabetic patients, the purpose of the present work was to (i) provide new data on the molecular changes induced by *in vitro* generated hydroxyl radicals on human insulin considering particularly changes in their aromatic residues: tyrosines, and phenylalanine (11), leading to the formation of dityrosine (12), conducing to protein aggregation, cross-linking, and fragmentation; (ii) evaluate the impact of the hydroxyl radical induced structural changes (11) on the biological action of insulin, measured by the glucose- ^{14}C utilization by human adipose tissue (radiorespirometry) in presence of human insulin, compared it to the chemically modified hormone and (iii) demonstrate the correlation of insulin oxidation with its chemical and biological consequences.

3. MATERIALS AND METHODS

3.1. Reagents

Human recombinant insulin (Humulin* R , Lilly) was obtained from Lilly Lab. México; H_2O_2 , CuSO_4 , trichloroacetic acid (TCA), were obtained from Sigma (St. Louis, MO, USA).

3.2. Hydroxyl (HO) radical generation

Hydroxyl radical generation was obtained from the Fenton reaction (10), using the following mixture: 5 mM H_2O_2 , 4.0 mM CuSO_4 , 5 IU of single human recombinant insulin (33.5 nanomol = 194.55 μg) in water. After different times of incubation (0-60 min) at 37°C , the reaction was stopped by the addition of 5 % TCA and centrifuged at 3500 rpm. The pellet was washed twice by centrifugation with 2.5 % TCA and finally re-suspended in one ml of tris-hydroxymethyl-aminometane- NaOH (90 mM) pH 8.5.

3.3. Dityrosine production

The suspension was utilized for protein-bound dityrosine production measured by fluorescence. The fluorescence excitation spectra of dityrosine was recorded from 280 to 370 nm using a PTI (Photon Technology International) spectrofluorometer, maximal emission was measured at 405 nm. The following control groups were used and all the evaluations were made comparing emission of samples and controls: a) insulin + H_2O , b) insulin + CuSO_4 , and c) insulin + H_2O_2 .

3.4. Subcutaneous fat tissue

All procedures were approved by Institutional Research and Ethics Board. The samples of abdominal adipose tissue were removed from healthy pregnant women (18-34 aged) during abdominal cesarean section deliveries. The tissues samples were placed in a flask containing cold

Krebs-Ringer buffer pH 7.4. The tissue were cut and weighted in order to obtain samples of 500 mg that were added to the main chamber of the Warburg flask.

3.5. Radiorespirometry

A Gilson differential respirometer adjusted to $37 \pm 0.1^\circ\text{C}$ was used for radiorespirometry (13). 5 ml Warburg flasks were used and the following materials were added: 0.1 ml of 2 M KOH (adsorbed into a 1 cm^2 filter paper) to the center well; 0.1 ml of 2 M perchloric acid to the side arm, and 0.5 ml (containing $500 \pm 20\text{ mg}$ of subcutaneous tissue) of the tissue samples into the main chamber. As substrate, 0.1 ml $\text{U}^{14}\text{C-D}(+)\text{glucose}$ (New England Nuclear CO_2), containing 5 μmol of the hexose (specific activity $0.02\text{ }\mu\text{Ci}/\mu\text{mole}$) were added. Incubations were carried out in presence of different insulin concentrations (0-1.5 IU) comparing the responses obtained utilizing native or oxidized hormone.

Finally, the volume was adjusted to 1 ml with Krebs-Ringer pH 7.4 in all flasks. After incubation (0 to 1.5 h) the reaction was stopped by pouring in the perchloric acid solution. Flasks were let undisturbed 30 min in order to allow the complete diffusion of CO_2 into the center well filter paper, the flasks were opened and substrate oxidation was calculated by counting the CO_2 trapped in the center well filter paper. A liquid scintillation spectrometer LKB Wallac 1209 Rackbeta was used for counting the $^{14}\text{CO}_2$. The flasks contents were homogenized with a Politron homogenizer, aliquots of homogenized tissues were used as reference parameter measuring dry weight (14).

3.6. Statistical analysis

Data were expressed as mean \pm SD. Data were analyzed by ANOVA and Bonferroni post 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. Quantification of dityrosines

For dityrosine characterization: we determined the optimal conditions for insulin oxidation as assessed by dityrosine concentrations related to the time of exposition to the oxidant medium. As shown in figure 1; the presence of dityrosine was detected at 320 nm of excitation since minute 2 of insulin oxidation. Figure 2 shows the values obtained utilizing the emission data obtained from 0 to 60 minutes, expressed as arbitrary units of fluorescence. The insulin oxidized by metal-catalyzed oxidation system ($\text{H}_2\text{O}_2/\text{Cu}^{2+}$) showed a time-dependent increase in dityrosine, whereas such an increase was not obtained when H_2O_2 or CuSO_4 were utilized independently (controls).

4.2. Radiorespirometry

Figure 3 shows the over-all production of $^{14}\text{CO}_2$ from U^{14}C -glucose utilized as substrate for human adipose tissue in presence of different concentrations of insulin (0-1.5 IU). The adipose tissue in absence of insulin produced

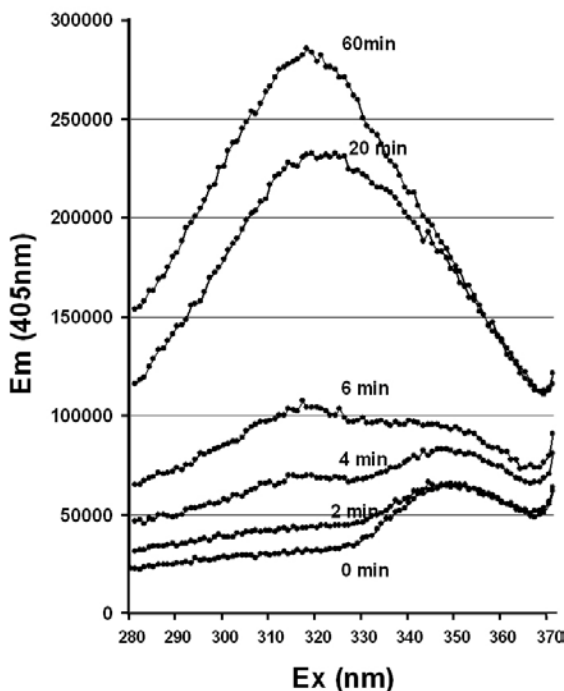


Figure 1. Fluorescence excitation spectra of insulin bound dityrosine. Maximal emission was measured at 405 nm.

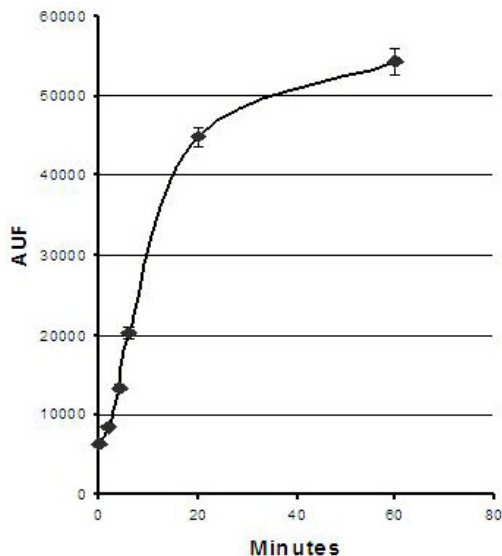


Figure 2. Time dependent formation of dityrosine. 301 ± 134 cpm/dry weight, due to the glucose utilization by nondependent-insulin cells; the addition of 0.1 or 1.0 IU of insulin increased 1.35 and 1.7 times respectively the glucose utilization by the tissue (Figure 3); the effect was not increased at higher insulin concentrations. On figure 4 we show the comparative effect of oxidized insulin at different times; the results obtained at 5 minutes showed that the biological effect of insulin on glucose utilization was 83 % less (from 662 to 336 cpm/mg dry weight) compared to native insulin. AUF (arbitrary units of fluorescence). Mean \pm SD, $n = 3$.

5. DISCUSSION

Oxidative damage to proteins (15) may be important *in vivo* both, directly (affecting the function of receptors, enzymes, transport proteins, generating new antigens that can provoke immune responses) and indirectly because it can contribute to secondary damage to other biomolecules (16).

Free radical attack on proteins can generate amino-acid radicals, which may crosslink or react with O_2 to give peroxy radicals. These may subtract H, triggering the formation of more free radicals and forming protein peroxides, which can decompose in complex ways, accelerated by transition metal ions, generating more radicals (17). Oxidation of amino acid residues such as tyrosine, leading to the formation of dityrosine, protein aggregation, cross-linking, and fragmentation, is an example of ROS damage *in vitro*. In contrast, evidence of the presence of such oxidatively damaged proteins *in vivo* and their possible clinical significance was still lacking until recently (12). In this work in order to demonstrate another structural chemical changes induced to the human insulin (11) and their possible relation with the oxidative stress present during the diabetes (18), we were able to characterize the formation of dityrosine-containing protein cross-linking products designated as advanced oxidation protein products (AOPP) (19) in the human insulin oxidized by metal catalyzed oxidation system (20), utilizing copper as transition metal (Figures 1,2); these structural changes added to those previously described (11) are related to a decrease of insulin biological activity, measured as glucose oxidation by adipose tissue (Figure 4).

Our results showed (Figure 1) an increase in fluorescence emission spectra related to oxidation time. Insulin was oxidized at different insulin/time ratios to induce differential oxidative modification. The dityrosine formation is detected after 2 minutes of oxidation and the emission increase at 4, 6, 20 and 60 min. The hydrogen peroxide in our system was unable to induce dityrosine formation due its quickly decomposition to water and atomic oxygen, however it is important to emphasize that the continuous flux of H_2O_2 produces oxidative modifications on erythrocytes and oxyhemoglobin. The main fragments formed were identified as oxidation products of tyrosine, including dopamine, dopamine quinone, and dihydroxyindol and dityrosine (21). Further release of these oxidation products including dityrosine was only seen after proteolytic degradation of the oxidatively modified protein.

Dityrosine crosslink formation of insulin might explain the observed insulin-aggregates formed during copper/ H_2O_2 -mediated oxidation. Similar complexes were obtained during copper/ H_2O_2 oxidation carried out utilizing the major element of neuronal cytoskeletons (Neurofilament-L) and known to be important for neuronal survival *in vivo* (22).

The biological effect of insulin was evaluated utilizing human insulin-dependent tissue, the viability of

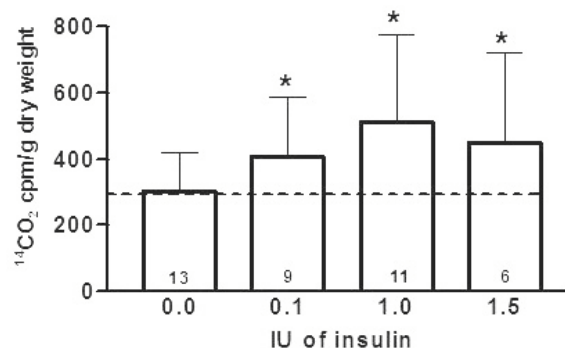


Figure 3. Generation of $^{14}\text{CO}_2$. The tissue generation of $^{14}\text{CO}_2$ /mg dry weight is shown as percent of increase of insulin biological activity. When 0.1, 1.0 or 1.5 IU was added the percent values obtained correspond to 135, 169 and 150 respectively. * $p < 0.05$ when compared with control group and corresponds to $^{14}\text{CO}_2$ generation by the tissue in absence of insulin.

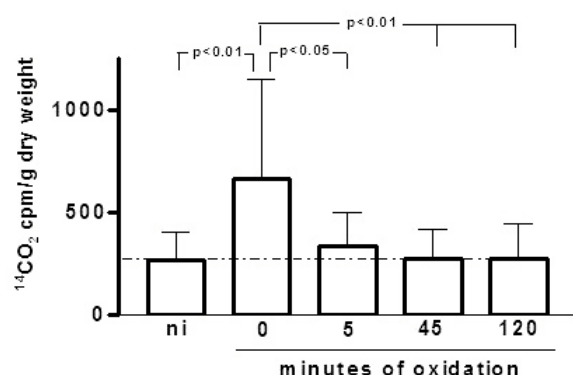


Figure 4. Biological activity. Effect on insulin biological activity due to their oxidation with the Fenton reaction by different time. The values are expressed as percent of basal activity. At 5 minutes the glucose utilization decreases to 125 % when compared to 246 % of increased activity of native insulin (0 minutes). At 45 and 120 minutes the values were 102 % compared to the generation of $^{14}\text{CO}_2$ generated by the non insulin dependent cells (ni) present in the tissue. The statistically values were calculated utilizing the cpm $^{14}\text{CO}_2$ /mg dry weight generated by adipose tissue utilization of U- ^{14}C glucose (0.02 mCi/M). ni vs 0 min $p < 0.01$; 0 vs 5 min $p < 0.05$; 0 vs 45 min $p < 0.01$; 0 vs 120 min $p < 0.01$. (ANOVA). Mean \pm SD. $n = 13$

the tissue and the presence of other cells different to adipocytes was shown in figure 2 and corresponds to $^{14}\text{CO}_2$ generation in absence of insulin. The addition of 0.1, 1.0 and 1.5 IU of insulin significantly increased the glucose utilization. The increase of glucose utilization induced by insulin is evident. This effect is drastically decreased within 5 minutes period oxidation of insulin, after this time, the glucose utilization disappeared (Figure 4). The structural modifications on insulin molecule including hydroxylation of tyrosine and phenylalanine residues, carbonyl exposition and pro-oxidant properties were previously demonstrated (11), and the new evidences of AOPPs formation detected as dityrosine, affect the metabolic function of insulin

related to the utilization of glucose by a insulin-dependent tissue.

In summary, we previously reported molecular changes in insulin (11) and those findings were confirmed in this study. The functional alteration of insulin caused by structural modification due to protein glycation (23), hydroxyl radicals and dityrosine formation may explain the diminished insulin efficiency in diabetes mellitus. These effects are related to the oxidative stress due to increased ROS generation including those produced by the hyperglycemic condition (24) observed during the insulin resistance and diabetes mellitus.

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7. REFERENTES

1. Krebs E. G: The growth of research on protein phosphorylation. *Trends Biochem Sci* 19, 439 (1994)
2. Cheeseman K. H & T. F. Slater: An introduction to free radical biochemistry. *Br Med Bull* 49, 481-493 (1993)
3. Halliwell B & M. Whiteman: Measuring reactive species and oxidative damage *in vivo* and in cell culture: how should you do it and what do the results mean?. *Br J Pharmacol* 142, 231-255 (2004)
4. Dreher D & A. F. Junod: Role of oxygen free radicals in cancer development. *Eur J Cancer* 32A, 30-38 (1996)
5. Regnström J, J. Nilsson, C. L. Tornvall, C. Landou & A. Hamsten: Susceptibility to low-density lipoprotein oxidation and coronary atherosclerosis in man. *Lancet* 339, 1183-1186 (1992)
6. Sies H: Oxidative Stress. Oxidants and Antioxidants. *Exp Physiol* 408, 289-291 (1997)
7. Simpson J. A, S. Narita, S. Giseg, S. Gebicki, J. M. Gebicki & R. T. Dean: Long-lived reactive species on free-radical-damage proteins. *Biochem J* 282(Pt 3), 621-624 (1992)
8. Lunes J, S. Brailsford, S. D. Hewitt, C. J. Morris & D. R. Blake: Free radicals: Are they possible mediators of IgG denaturation and immune complex formation in rheumatoid arthritis. *Int J Immunotherapy* 3, 39-43 (1987)
9. Silvester J.A, G. S. Timmins & M. J. Davies: Photodynamically generated bovin serum albumin radicals: evidence for damage transfer and oxidation at cysteine and tryptophan residues. *Free Radic Biol Med* 24, 754-766 (1998)

10. Ortega C, A. M. Guzmán-Grenfell & J. J. Hicks: Oxidation of PMSG by oxygen-free radicals, change its structure, affecting the hormonal activity. *Mol Reprod Dev* 52, 264-268 (1999)
11. Olivares-Corichi I. M, G. Ceballos, C. Ortega-Camarillo, A. M. Guzman-Grenfell & J. J. Hicks: Reactive Oxygen Species (ROS) induce chemical and structural changes on human insulin *in vitro*, including alterations in its immunoreactivity. *Frontiers Biosci* 10, 838-843 (2005)
12. Heinecke J. W, W. Li, H-L. Daehnke III & J. A. Golstein: Dityrosine, a specific marker of oxidation, is synthesized by the myeloperoxidase-hydrogen peroxide system of human neutrophils and macrophages. *J Biol Chem* 268, 4069-4077 (1993)
13. Hicks J. J, L. Rojas & A. Rosado: Insulin Regulation of Spermatozoa. *Endocrinology* 92, 833-839 (1973)
14. Hicks J. J, N. Pedrón & A. Rosado: "Modification of human spermatozoa glycolysis by cAMP estrogens and follicular fluid". *Fertil. Steril* 23, 886-893 (1972)
15. Halliwell B & M. Whiteman: Measuring reactive species and oxidative damage *in vivo* and in cell culture: how should you do it and do the results mean?. *Br J Pharmacol* 142, 231-155 (2004)
16. Chevion M, E. Berenshtein & E. R. Stadman: Human studies related to protein oxidation: protein carbonyl content as a marker of damage. *Free Radic Res* 33(Suppl), S99-S108 (2000)
17. Dean R. T, S. Fu, R. Stocker & M. J. Davies: Biochemistry and pathology of radical-mediated protein oxidation. *Biochem J* 324(Pt 1), 1-18 (1997)
18. Ceriello A, N. Bortolotti, E. Motz, A. Crescentini, S. Lizzio, A. Russo, L. Tonutti & C. Taboga: Meal-Generated oxidative stress in Type 2 diabetic patients. *Diabetes Care* 21, 1529-1533 (1998)
19. Witko-Sarsat V, M. Friedlander, C. Capeilleère-Blandin, T. Nguyen-Khoa, A.T. Nguyen, J. Zingraff, P. Jungers & B. Descamps-Latscha: Advanced oxidation protein products as a novel marker of oxidative stress in uremia. *Kidney Int* 49, 1304-1313 (1996)
20. Kato Y, N. Kitamoto, Y. Kawai & T. Osawa: The hydrogen peroxide/copper ion system, but not other metal catalyzed oxidation systems, produces protein-bound dityrosine. *Free Radic Biol Med* 31, 624-32 (2001)
21. Giulivi C. & K. J. Davies: Mechanisms of the formation and proteolytic release of H₂O₂-induced dityrosine and tyrosine oxidation products in hemoglobin and red blood cell. *J Biol Chem* 276, 24129-36 (2001)
22. Kim N. H. & J. H. Kang: Oxidative modification of neurofilament-L by copper-catalyzed reaction. *J Biochem Mol Biol* 36, 488-92 (2003)
23. McKillop A. M, M. H. Mooney, P. Harriott, P. R. Flatt & F. P. O'Harte: Evaluation of glycated Insulin in Diabetic Animals using Immunocytochemistry and Radioimmunoassay. *Biochem Biophys Res Comm* 286, 524-528 (2001)
24. Hunter S. J, A. C. Boyd, F. P. O'Harte, A. M. McKillop, M. I. Wiggam, M. H. Money, J. T. McCluskey, J. R. Lindsay, C. N. Ennis, R. Gamble, B. Sheridan, C. R. Barnett, H. McNulty, P. M. Bell & P. R. Flatt: Demonstration of glycated insulin in human diabetic plasma and decreased biological activity assessed by euglycemic-hyperinsulinemic clamp technique in humans. *Diabetes* 52, 492-498 (2003)

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