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PHARMACOLOGICAL SPARING OF PROTEIN AND GLUCOSE IN BURN
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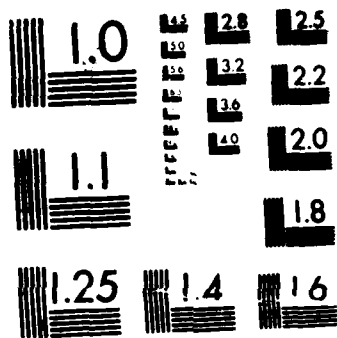
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INJURY AND/OR SEPSIS

ANNUAL PROGRESS REPORT

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January 15, 1987

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FOREWORD

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- (1). In a very comprehensive study conducted during 1983-1985, we investigated the roles of insulin and glucagon in mediating the changes in glucose and alanine kinetics during the hypermetabolic response to burn injury.

Twelve experiments were performed in 10 patients with burn size ranging from 44-90% TBSA. The study consisted of two experimental protocols and patients from the study population were randomly assigned to either protocol. The aim of protocol 1 was to measure the basal rates of glucose production and alanine flux and to assess the response to an acute and simultaneous suppression of insulin and glucagon release induced by the infusion of somatostatin. In protocol 2 the response of the same parameters was assessed during the selective lowering of glucagon concentration by infusing insulin along with somatostatin in order to maintain basal insulin levels constant. In all experiments, primed-constant infusions of the isotopes 6, 6-d₂-glucose and 3-¹³C-alanine were used to measure glucose and alanine kinetics.

Our results showed that the basal rate of glucose production and alanine flux were significantly elevated in all patients when compared with the values of normal human volunteers. Lowering both insulin and glucagon simultaneously caused an insignificant reduction in glucose production, but plasma glucose concentration increased significantly ($P < 0.01$), because of a significant reduction ($P < 0.05$) in glucose clearance. Alanine flux and the concentration of all plasma amino acids increased significantly ($P < 0.05$) above basal values.

Selectively lowering glucagon concentration while maintaining basal insulin concentration constant, caused a significant reduction in glucose production ($P < 0.05$) and exogenous glucose was infused to maintain euglycemia. Alanine flux and plasma amino acid concentrations remained unchanged.

From these results we concluded that in severe burn injury, hyperglucagonemia stimulates an increased glucose production, since the lowering of glucagon concentration in both protocols caused a decrease in glucose production. Basal insulin plays an important role in suppressing glucose production, stimulating glucose clearance, and regulating the release of amino acids from peripheral tissues, implying that it inhibited protein catabolism. Finally, the selective lowering of glucagon while maintaining basal insulin constant, normalized glucose kinetics.

- (2). As a prerequisite for our proposal to study, "The effect of increasing the plasma concentration of insulin above basal level, while maintaining euglycemia, on the rate of protein breakdown and the rate of synthesis of albumin, fibrinogen and fibronectin", during the upcoming year, we had to develop several new biochemical procedures and perform several control experiments in normal human volunteers. The following methods were developed in order to measure plasma protein synthetic rates.

We infused ^{15}N -glycine in order to calculate the fractional synthetic rates of the plasma proteins from the rate of incorporation of the labeled amino acid into the specific proteins per unit time. Urinary hippuric acid enrichment was used to estimate the enrichment of their precursor (glycine pool). The absolute protein synthetic rates were then calculated by multiplying fractional synthetic rate by the total intravascular protein mass (plasma volume x concentration). In order to achieve this, all 3 proteins had to be isolated from plasma in a pure form, hydrolyzed in order to release their individual amino acids and the glycine fraction extracted in a pure form for mass spectrometric analysis. Urinary hippuric acid also had to be extracted for mass spectrometric analysis and the plasma concentration of the proteins had to be determined.

Plasma Protein Concentration:

Plasma fibrinogen concentration was determined on a BBL Fibrometer (BBL Fibrosystem, Division of Becton, Dickinson and Company, Cockeysville, MD).

Plasma albumin concentration was determined colorimetrically using a Sigma Diagnostic Kit (Sigma Chemical Co., St. Louis, MO), and plasma fibronectin concentration was determined by a turbidimetric immunoassay which measured a change in absorbance at 334 nm when 0.02 cc of plasma sample was reacted with 0.01 cc of fibronectin antisera in 1 cc of phosphate buffer, pH 7.4, medium.

Isolation of Plasma Proteins:

Fibrinogen was isolated by treating plasma with 0.5 M calcium chloride solution (0.5 ml to 1 ml plasma) before clotting with thrombin 20 u/ml, (Sigma Chemical Company). The clot was separated from serum by centrifugation at 5,000 g at 4°C for 20 minutes.

Fibronectin was isolated from the serum by affinity chromatography using a gelatin-sepharose column (Affi-gel gelatin, Biorad).

Albumin was separated from the protein-containing fractions of the eluant from the fibronectin isolation column. The fractions were pooled and pumped through a column of Blue Sepharose CL-6B (Pharmacia). Albumin was eluted with a 0.05 M Tris/HCl buffer, pH 7.0 containing 1.5 M KCl. After concentration by lyophilization, the albumin was further purified by extracting with alcohol-TCA solution by the method of Korner and Debro and dialyzed for 24 h against repeated changes of distilled water.

The albumin, fibrinogen and fibronectin were hydrolyzed in vacuo at 110°C for 24 h with 6 N HCl (5 mg/ml) in order to release the protein-bound amino acids. The protein hydrolysate was then passed through columns of acid washed celite "to clear" it of carbon particles and humin.

Hippuric acid was separated from urine as follows: An aliquot of urine was cleaned with charcoal, acidified, and extracted with methylene chloride. The propyl ester of the hippuric acid residue was then made by the addition of propanolic hydrogen bromide, followed by heating. The isotopic enrichment was measured on a Hewlett-Packard 5985 GCMS system.

Measuring Isotopic Enrichment of Protein Bound Glycine:

The isotopic enrichment of fibronectin-bound glycine was measured by GCMS on its N-acetyl propyl ester (NAP) derivative. The amino acids of the fibronectin hydrolysate solution were further purified by desorption with NH_4OH from a column of Dowex 50-W-X8 (200/400 mesh) hydrogen form cationic resin before derivatization. The NAP esters were prepared by standard procedures and the enrichment of glycine measured by GCMS analysis.

The isotopic enrichments of albumin and fibrinogen-bound glycine were measured by isotope-ratio mass-spectrometry. Glycine was separated from the hydrolysates by ion exchange chromatography on a Beckman 119 Amino acid analyzer (Beckman Instruments, Inc., Irvine, CA). The glycine containing fractions were pooled and further purified by completely isolating from tracer contamination with alanine on a column of Dowex 50-W-X8 (200-400 mesh) cationic resin (H^+ form) and eluting with 0.5 M sodium citrate buffer, pH 3.5. The glycine containing fractions were pooled, concentrated, and glycine-N liberated as ammonium by digestion with concentrated sulphuric acid using a Tecator Digestion System (Tecator, Hoganas, Sweden). The liberated ammonia was evolved by alkaline steam distillation in a Kjeltac System 1002 Distilling unit (Tecator, Hoganas, Sweden) and trapped in 0.05 N H_2SO_4 and analyzed for ^{15}N -enrichment in a triple-inlet, 6", triple-collector isotope-ratio mass-spectrometer (IRMS) (Nuclide 3-60 rms).

To date we have thoroughly worked out all aspects of the experimental protocol, from the initial stages of isotope infusion, to the final processing and analysis of samples. More than six studies have been completed in normal volunteers in order to obtain basal protein synthetic rates, which will be used as controls for the basal values obtained in the burn patients.

(3). We have also completed a series of control studies in postabsorptive normal human volunteers, in which the effect of different elevations in plasma insulin concentration above basal level (while maintaining euglycemia) on the rate of protein breakdown was investigated. The rate of protein breakdown was measured in the basal state and during the administration of insulin at rates of 15, 40, 120 and 240 $\text{mU/M}^2/\text{min}$ for 2 hour intervals, which resulted in increases in plasma insulin concentrations of 30, 60, 200 and 700 uU/ml . During this multistage insulin clamp, euglycemia was maintained by infusing 20% glucose. Stable isotope of leucine was infused throughout the duration of the experiment in order to measure leucine kinetics and the absolute rate of protein breakdown was calculated from the plasma flux of leucine.

Our results so far show that for each step-increase in insulin concentration there is a corresponding decrease in leucine flux and oxidation.

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