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EFFECT OF INSULIN ON ASCORBIC ACID UPTAKE BY HEART ENDOTHELIAL CELLS: POSSIBLE RELATIONSHIP TO RETINAL ATHEROGENESIS

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Summary

Increasing concentrations of insulin were found to increase transport of radioactive ascorbic acid into fetal bovine heart endothelial cells (FBHE). A linear relationship was found between the log of the insulin concentration (range 0 μU/ml to 400 μU/ml) and the uptake of ascorbic acid quantified as dpm/μg protein. Evidence has accrued which relates ascorbic acid to atherogenesis by its possible effect on preventing the breakdown of the glycosaminoglycan matrix of the intimal layer of the artery. Since insulin was found to increase ascorbic acid uptake, any compound, like glucose, that competes for the carrier mechanism may, if present in high enough concentrations, competitively inhibit ascorbic acid transport into the cell. The hyperglycemia and inadequate insulin production associated with diabetes mellitus may cause an ascorbic acid deficiency within the cell. This deficiency would lead to intimal matrix breakdown with subsequent increase in atherogenesis. The microangiopathies associated with diabetes and with the aging process itself may be related to this mechanism.

Recent evidence has been reported detailing the effects of ascorbic acid on atherogenesis, therefore, its transport into the cell becomes of primary interest. According to the ground substance hypothesis, endothelial cells secrete an extracellular matrix composed of glycosaminoglycans (GAG) and collagen (1). The breakdown of this matrix causes the intima to lose it selective permeability. Ascorbic acid serves to prevent the breakdown of this matrix by interacting in glycosaminoglycan metabolism. Researchers (2,3) have shown that with increasing atherosclerosis there is a decreasing concentration of GAG in the matrix. Other work (4) has shown that matrix changes associated with atherosclerosis parallel ascorbic acid deficiency. Animal studies (5,6,7) also have demonstrated that ascorbate administration to animals with experimentally induced atherosclerosis reduced the disease state.

There is an indication that ascorbic acid serves a role in preventing atherogenesis. Hence, study of its transport into the cell and subsequent effect on matrix GAG is of interest. Mann (8) hypothesized that humans require insulin for the transport of vitamin C into cells. He further stated that low levels of insulin, such as would occur in diabetes mellitus, or in impairment of insulin function, would cause a "local scurvy" in insulin sensitive tissues, leading to

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fragility of the arterial wall. It also has been shown by Mann (9) that, in the red blood cell, a number of sugars including D-glucose, D-mannose and D-xylose strongly inhibit the uptake of dehydroascorbic acid, the transportable form of ascorbic acid in the body. Based on this finding of glucose competition with ascorbic acid for the transport system, hyperglycemia, which occurs in diabetes mellitus, would be expected to cause an ascorbate deficiency within the cell. This deficiency in the arterial tissue may lead to degeneration of the ground substance and vascular wall fragility. These resultant lesions have been related to the various microangiopathies that are frequently associated with diabetes such as retinopathy, nephropathy, and macroangiopathies of the coronary, cerebral and peripheral blood vessels. Since glucose competitively inhibits ascorbic acid transport, a large intake of ascorbic acid may overcome this competition. This could possible prevent or reduce the progression of the microangiopathies that are associated with hyperglycemia.

Ralli and Sherry (10) have found that injection of insulin into normal and depancreatized dogs caused a decrease in both the plasma levels and urinary excretion rates of ascorbic acid. They concluded that ascorbic acid is somehow redistributed in the body from the plasma into the body tissues or fluids in response to insulin.

Verlangieri et al. (11) have shown that L-ascorbic acid reduces the lesions of rabbit induced atherogenesis relative to controls. Furthermore, it has been shown (7) that the mechanism of this reduction is due to the inhibition of GAG degradation. Accordingly, this study was designed to demonstrate L-ascrobic acid's insulin dependent transport into endothelial cells. The hypothesis then being if L-ascorbic acid requires insulin for transport, hyperglycemia may cause saturation of the transport system with glucose and cause increased degradation of the GAG matrix with a parallel increase in atherogenesis.

Most of the work dealing with ascorbic acid transport has been done on issue or cells that are not sensitive to insulin such as erythrocytes and nerve issue. This study utilized bovine endothelial cells which are insulin dependnt for glucose transport and which contribute to the intimal glycosaminoglycans.

Materials and Methods

Initial Culturing. FBHE (Fetal bovine heart endothelial; Bos Taurus) cells were obtained from the American Type Culture Collection. All cell culture work was carried out using aseptic technique in a laminar flow hood. The contents of the stock vials were each diluted 1:12 using Ham's F-12 growth media (pH 7.4). Four ml of the resulting solutions were placed into each of six 25 cm^2 T-flasks. The cells were grown in an incubator at 37°C. The following day the cells had attached to the bottom of the flasks and were washed twice (4 ml aliquots each time) with Hanks' Balanced Salt Solution (HBSS), pH 7.4. New growth media was added (4 ml) as was fibroblastic growth factor (FGF, Collaborative Research) (100 ng/ml). The cells were maintained with bi-weekly washing and changing of growth media at which time the cells were washed with 4 ml aliquots of HBSS and 4 ml of fresh growth media added. Growth factor was added for three weeks or until the cells reached confluency (determined by phase contrast microscopy and paralled T-flask silver nitrate staining) (12).

Digestion and Replating (first passage). The cells were first washed twice with 4 ml of HBSS, (minus calcium and magnesium), and then 2 ml of trypsin (0.25%, 1% chicken serum) were added (13) to each of the flasks. The flasks were gently shaken several times and then incubated for 15 minutes at 37°C. Two ml of cold HBSS were added to stop the digestion and the flasks were again shaken to remove any cells that were still attached to the bottom of the flask. The contents of

the flasks were transferred to 15 ml centrifuge tubes and centrifuged for 3 minutes. The supernatant was poured off and the cells were resuspended in 12 ml of growth media and transferred into respective 75 cm^2 T-flasks. Again, FGF was added until confluency was reached.

Experimental Design. Two of the large 72 cm^2 T-flasks were digested (second passage) as described above two weeks prior to the study. Seven ml of trypsin solution were added to digest the cells and an equal amount of cold HBSS was added to stop the digestion. The cells from each flask were taken up into 36 ml of growth media and 4 ml were added to each of nine 25 cm^2 T-flasks. When the cells were first replated, they were round and compact in shape. Four days after replating, the cells started to "spread out," sending forth cytoplasmic projections that resembled pseudopodia and the cells became stellate in appearance. As the cells approached confluency, they became polygonal in shape. At confluency, they exhibited contact inhibition and were tightly packed assuming a more rounded shape. The 18 T-flasks were divided into 6 groups containing three flasks each and treated as shown in Table I.

TABLE I

Experimental Design

Group[a]	μU Insulin/ml	^{14}C-AA (mg %)
1 (control)	0	1.225
2	100	1.225
3	400	1.225
4	1600	1.225
5	3200	1.225
6	4000	1.225

[a]Each group run in triplicate

Insulin was obtained from Eli Lily Co. (beef, U-100 R, 1000 units/cc). Radioactive ascorbic acid was purchased from New England Nuclear and C^{14}-labeled at C-1 (specific activity 9.0 mCi/mmol.). 4.9 mgs of labeled C^{14}-ascorbic acid were dissolved in 1 ml of sterile, distilled water. All dilutions were made in Ringer's Injection, U.S.P. (Abbott Laboratories) buffered to pH 7.62 with Neut (4% sodium becarbonate solution, Abbott Laboratories).

The concentration of ascorbic acid was chosen as 1.225 mg% to reflect physiological levels (14). Insulin levels were chosen to approximate increasing *in vivo* physiological ranges (15). A pH of 7.62 was used as in previous studies (16,17,18).

Growth media was withdrawn from all flasks and the cells washed 2 x with HBSS then three mls of the labeled ascorbic acid-insulin solutions were added to the T-flasks and incubated at 37°C for 80 minutes. After 80 minutes, the media was removed. The cells were washed twice with HBSS-(4 ml) to remove any isotope that may have remained, and verified as equal to or less than background in last wash. The cells were then digested with trypsin as previously

described. Two mls of ice cold HBSS were added to stop the digestion. The flasks were gently shaken and each entire contents centrifuged. Two fractions resulted: 1. the cell fraction (pellet) and 2. the adhesion fraction (the supernatant). The supernatant was poured off and one ml of distilled water was added to the cell fraction samples and sonicated for 1 minute (Sonifier Cell Disrupter, Fine probe, Setting #7). Radioactivity was quantified in the cell fraction by adding 0.5 ml of the sample to 10 ml of scintillation cocktail (Instagel, Packard). The samples were counted 3 times each for five minutes in a Packard Model 3255 liquid scintillation counter. The counter was equipped with a minicomputer (Monroe, Scientist 325) and was programmed for quench correction, which resulted in direct conversion of cpm to dpm. The remaining 0.5 ml of each sample was analyzed for protein in duplicate 0.1 ml aliquots by the method of Lowry et al.

Results

The C^{14} activity for the cell fraction is reported as dpm/µg cell protein. The mean values and ranges for each of the six groups are shown in Table II. The data shows increases in cell uptake of ascorbic acid with increasing levels of insulin.

TABLE II

Effect on Insulin Concentration on Cell C^{14}-Ascorbic Acid Uptake

Group	µU Insulin/ml	dpm C^{14}-AA µg cell protein	p[a]
1	0	21.8 ± 4.9	-
2	100	32.3 ± 8.6	<.10
3	400	44.6 ± 0.1	<.01
4	1600	47.5 ± 6.1	<.005
5	3200	62.2 ± 23.5	<.025
6	4000	51.7 ± 3.3	<.005

[a]Significance compared to Group 1 (control), Two-tailed T-test

Discussion

It is apparent from the data that insulin increases the transport of ascorbic acid into the cell. With increasing concentration of insulin, there is increased uptake of ascorbic acid. There is seen a leveling off of ascorbic acid uptake at insulin levels greater than 400 µU/ml. Podskalny (17) found that cooperative interactions occur with insulin at physiological concentrations, and has noted negative cooperativity (i.e. insulin binding and affinity for the receptor site decreases) when concentrations exceed the physiological limit. Oleafsky (16) has calculated the maximum number of insulin molecules that that can be bound to a mononuclear leukocyte from a normal patient to be 2300 ± 140 molecules. He also noted that as the total insulin concentration increases, the percent bound decreases as a result of negative interaction. Further evidence from the literature to support the data has been advanced by Ralli and

Sherry (19) who noted that insulin action is not proportional to the arthmetic dose but to the logarithm of the dose. The leveling off above 400 μU insulin/ml may in part be due to negative cooperativity effect.

In a similar uptake study in media containing a 180 mg% glucose concentration there was approximately a five-fold decrease in ascorbic acid uptake. This is most likely attributable to the competition of glucose with ascorbic acid for the insulin receptor-site complex. Glucose apparently has a higher affinity for the insulin receptor-site complex than does ascorbic acid.

Since ascorbic acid seems dependent on insulin for an active transport, any condition which results in a reduction in insulin production or action or hyperglycemia (which causes competitive inhibition for the transport complex) should result in an ascorbate deficiency in the cell. Matrix breakdown and atherogenesis may result. Retinopathy, a complication of diabetes mellitus, is a result of atherosclerosis of the retinal circulatory system. Perhaps increased intake of vitamin C would prevent or even possible reverse the lesions associated with retinal atherosclerosis by saturating the plasma with ascorbic acid, and, therefore, overcoming the glucose competition for the insulin-receptor transport complex.

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