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Human Insulin Modulation of *Escherichia coli* Adherence and Chemotaxis

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Abstract: *Escherichia coli* exhibited increased hydrophobicity and mannose-resistant epithelial cell adherence after growth in the presence of human insulin (2 μU mL^{-1} or 200 μUmL^{-1} insulin, respectively) with glucose (100 mg dL^{-1}). Capsule production and hemagglutination were unaffected by insulin and glucose. Chemotactic attraction to glucose as compared to insulin or glucose alone was enhanced by the presence of insulin. Insulin alone (200 μU mL^{-1}) was a chemorepellent and inhibited flagellar tethering to glass. These findings indicate that human insulin can modulate *E. coli*'s expression of factors associated with pathogenesis in a manner that is modifiable by the presence of glucose.

Key words: Human insulin, uropathogenic *E. coli*, adherence, chemotaxis

INTRODUCTION

Escherichia coli is the most common etiologic agent of bacteremia, urinary tract infections (UTI) and acute pyelonephritis[1]. Any organism which causes systemic infections is exposed to a variety of host factors including hormones. The most prevalent and pervasive of these hormones is insulin. Previous studies determined that *E. coli* alters its growth kinetics in response to human insulin[2]. This insulin mediated change in doubling time is glucose dependent and reminiscent of quorum sensing, i.e., population size, dependent. However, other than affecting growth rates, insulin's effect on *E. coli* phenotype is unknown. The focus of this study was to determine the effect human insulin has on expression of factors reported to be associated with *E. coli*'s ability to colonize, i.e., adherence-related colonization factors and motility.

MATERIALS AND METHODS

Bacterial isolates and growth conditions: *E. coli* ATCC 25923 a highly stable *E. coli* K12 routinely used for quality control testing was grown as previously described[2] in peptone (1.0 gm dL^{-1}) yeast nitrogen base broth (PYNB) containing insulin at 2, 4, 20 and 200 μU mL^{-1} (Humulin® R, Eli Lilly and Co., Indianapolis, IN) and/or glucose (0.1, 0.5, 1.0, 2.0 and 5 gm dL^{-1}) unless otherwise indicated.

Quorum sensing: Autoinducers I and II were measured as described by Surette *et al.*[3] with *Vibrio harveyi* strains generously provided by Bonnie Bassler (Princeton University, NJ). Positive controls were filtrates from *Vibrio harveyi* BB152 and BB120 grown in medium with (200 μU mL^{-1}) and without insulin and/or glucose (0.2 gm dL^{-1}). *E. coli* were grown in Mueller-Hinton broth containing insulin (200 μU mL^{-1}) and/or 0.2 gm dL^{-1} glucose. Mid-log, early stationary and late stationary growth phase (24 hr) cells were pelleted (12,000 X g, 10 min, RT) and the supernatant filter sterilized then stored (-70°C) until use. *V. harveyi* BB170 and BB886 (O/N culture, 30°C, 1:5 X 10^3 dilutions) luminescence (Coleman luminometer) was measured hourly after exposure to cell filtrate. Negative controls contained sterile medium.

Surface hydrophobicity: Surface hydrophobicity was determined as previously described[4]. *E. coli* were grown overnight (37ºC) in PYNB with and without insulin (2, 4, 20 and 200 μU mL^{-1}) and/or glucose (0.1, 0.5, 1.0, 2.0 and 5.0 gm dL^{-1}) in flat bottom tissue culture plates (150μL $well^{-1}$). After careful washing (4X: PBS; pH 7.0), the plates were air dried, stained with crystal violet (50 ml dL^{-1} v/v PBS; 20-30 min; 25^0 C) and bacterial adherence to plastic measured (EIA reader, Dynatech Laboratories, Inc., Chantilly, Virginia; 590 nm).

The production of acidic polysaccharide capsule was determined essentially as described for surface hydrophobicity[4] except that bacteria were stained with Alcian blue (0.3 gm dL^{-1} PBS, 30 min, room temperature; EIA reader, Dynatech Laboratories, Inc., Chantilly, Virginia; 450 nm).

Epithelial cell adherence: Human buccal and uroepithelial cells were obtained and stored until use as previously described[5-7]. The adherence assay was performed as described by Leffler *et al.*[8]. *E. coli* (0.05, Abs_{540nm}) grown in Mueller-Hinton broth alone (growth control) or with insulin (200 μU mL^{-1}) and/or 0.2 gm dL^{-1} glucose (St. Louis, MO) were washed and suspended (10^8 CFU mL^{-1}) in PBS with and without 2 gm dL^{-1} mannose. One ml of bacterial suspension was

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mixed and incubated (30 min, 37ºC, 200 rpm) with an equal volume of uroepithelial or buccal cells (10^5 cells mL^{-1}). The negative control was epithelial cells with 1 ml of PBS alone. After incubation, unattached bacteria were removed by centrifugation (250 X g, 10 min; 4X). The suspension was then dried, fixed and stained with crystal violet. The number of *E. coli* adherent to 50 epithelial cells $assay^{-1}$ was counted.

Hemagglutination: Defibrinated sheep erythrocytes (Remel, Lenexa, KS) were washed twice in PBS and suspended to a final concentration of 0.5 cells dL^{-1}. *E. coli* (50 μL^{-1}, 10^{10} cells mL^{-1} PBS with and without 2 gm dL^{-1} mannose) grown as described above for epithelial cell adherence were mixed with an equal volume of erythrocytes, then incubated at room temperature for 2-4 hours before reading[9].

Chemotaxis: Chemotaxis was assessed essentially as described by Adler[10,11] with the geotactic effects controlled by placement of capillary tubes in 5ml beakers containing 0.5 mL^{-1} of bacteria suspension instead of U-tubes. Mid-logarithmic growth phase cells (37 ^{0}C, shaken; OD_{590} of 0.4) were harvested by centrifugation at 6,000 3 X *g* for 3 min. The pellet was washed twice in chemotaxis medium (10 mM K_2HPO_4, 0.1 mM EDTA) pH 7.0 then adjusted to a final concentration of 7 x 10^6 CFU mL^{-1}.

Analysis of free-swimming behavior: Swimming behavior of cells suspended in PYNB (OD_{590} of 0.1) with and without insulin and/or glucose, as described above, was assessed by bright-field microscopy (Petroff-Hauser counting chamber) and the extent of translocational movement and tail-spinning (flagellar tethering) determined.

Statistical evaluation: All assays were done in triplicate and repeated at least twice. Whenever possible, experiments were coded and performed in a blinded fashion. Analysis of variance was used to determine differences between experimental conditions with $P < 0.05$ considered significant. If statistical significance was found, a Tukey-Kramer post-hoc analysis was applied (Instat, GraphPad Software, San Diego, CA).

RESULTS AND DISCUSSION

Human insulin in the presence of glucose affects expression of colonization factors. Insulin with glucose significantly ($P < 0.05$) enhanced adherence, biofilm formation and chemotactic response to glucose as compared to medium/buffer alone or medium/buffer with glucose (Table 1). The adherence to epithelial cells or erythrocytes was not affected by the presence of mannose. Microscopically, mid-logarithmic *E. coli* cells grown in insulin and glucose (200 $\mu U\ mL^{-1}$ insulin; 200 mg dL^{-1} glucose) were elongated, appeared tethered by their flagella to glass and exhibited clockwise and counterclockwise rotation[12-14]. With the exception of chemorepulsion, insulin in the absence of glucose exhibited no other measurable effect on the virulence factors examined. Similar to the effects of insulin and glucose on growth rate[2], the effects of insulin and glucose on virulence factor expression were concentration specific. Capsule production and hemagglutination were unaffected by the presence of insulin with or without glucose at all concentrations and combinations tested.

Some of the insulin effects measured may be due directly, or indirectly to the presence of homoserine lactone (AI-1) or cyclic borate diester (AI-2) which are autoinducers of quorum sensing and altered virulence factor production[3,15,16]. Addition of cell-free culture fluid of *E. coli* strains to either *V. harveyi* reporter strain BB170 (autoinducer-2) or BB 886 (autoinducer-1), showed no light production under conditions containing glucose and/or insulin indicating that under these growth conditions acyl-homoserine lactone autoinducers are not produced by *E. coli*.

Prior studies show that *E. coli* K12's microbial insulin has chemical, immunological and biological properties similar to that of mammalian, worm and protozoan insulin, although basic local alignment search tool (BLAST) analysis comparing human insulin and the *E. coli* genome indicated a lack of significant alignments which is not entirely surprising since human insulin is post-translationally processed[17-19]. In addition to affecting growth characteristics[2], insulin with glucose also enhances adherence to epithelial cells, which is considered to be the first step in colonization of host mucosal surfaces. It is well documented that *E. coli* grown in medium with glucose exhibits an increased ability to adhere to uroepithelial cells[20]. Our findings indicate that insulin augments the effect of glucose thereby boosting epithelial cell adherence. This adherence may be mediated by a variety of adhesive factors[20-27] which are affected by insulin including fimbriae (P-pilus, because of mannose-resistance) and/or hydrophobic interactions. Microscopic examination of the motile cells also showed a tethering of cells to glass via their flagella in the presence of insulin and glucose but not insulin alone. Increased adherence to glass, a negatively charged surface, is reported to be indicative of ionic interactions and increased bacteria cell surface positive charge[28]. Increased positive surface charge has been correlated with an increased ability to interact with mammalian cell surfaces which carry a negative charge[29]. The tethering and rotational movement may also be reflective of localized changes in flagella composition resulting in electrostatic changes. However, while flagella have been reported to mediate adherence in enteropathogenic *E. coli*[30] its role in *E. coli* K12 cellular adherence has not been established.

Table 1: Altered expression of virulence-associated factors by insulin and/or glucose

Virulence Factors	Media[a] Control	Media & Glucose (100mg dL^{-1})	Media and Insulin[b]	Media with Glucose (100mg dL^{-1}) and Insulin
Cell adherence [c]	10.1 ± 0.26	60.6 ± 2.14[e]	7.0 ± 0.4[b]	93.4 ± 7.5 (200 µU of insulin) [de]
Hydrophobicity	5 ± 0.2	10.5± 0.1[e]	5.5± 0.3[b]	14.5 (2µU insulin) [de]
Chemotaxis	280 ± 23	710 ± 56[e]	81 ± 5 [deg]	1210 ± 65 (200µU insulin) [def]

[a]– PYNB and Mueller Hinton. All assays were done in triplicate and repeated twice.
[b]–2, 4, 20, or 200 µU of insulin
[c]– number of *E. coli* /epithelial cell ± S.D.
[d] significantly different from glucose alone; $P < 0.05$
[e] significantly different from media alone; $P < 0.05$
[f] significantly different from insulin alone; $P < 0.001$
[g]200 µU insulin was the effective insulin concentration

Flagella can also play another role in uropathogenesis. For *Proteus mirabilis* motility has been shown to contribute to virulence in ascending UTIs[31]. Whether motility and directional translocation play a similar role in the establishment of *E. coli* urinary infections remains to be determined. It is interesting that in the absence of glucose, an energy source, insulin acts as a chemo-repellent. However, in the presence of glucose insulin has the reverse effect, i.e., enhancing chemo-attraction to glucose. This effect of insulin on glucose chemotaxis indicates that the regulatory circuitry for glucose metabolism and chemotaxis may be more complex than previously thought[32].

Based on the observations made in this study, human insulin appears to differentially regulate expression of virulence factors dependent on the presence of glucose. In the absence of glucose, insulin appears to act in a warning mode as a chemo-repellent seemingly signaling an inhospitable environment for colonization and promoting a planktonic population. In contrast, with glucose present insulin appears to signal a welcoming environment for colonization and long term survival by enhancing expression of adherence properties. This control of environmental adaptation would be crucial for environmental survival perhaps functioning through modulation of the CsrA/CsrB and BarA/UvrY systems which are part of the glucose-mediated regulatory circuitry for virulence factor expression, including biofilm formation[33]. The possible role this insulin-mediated switching of virulence factor expression plays in asymptomatic bacteriuria vs. the higher rate of urinary tract infections occurring in patients with type 2 diabetes and in females with gestational diabetes as compared to type 1 diabetics remains to be determined.

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