Identification and characterization of an ascorbic acid transporter in human granulosa–lutein cells

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Ascorbic acid serves a vital role as a pre-eminent antioxidant. In animals, it has been shown to be concentrated in granulosa and theca cells of the follicle, in luteal cells of the corpus luteum, and in the peripheral cytoplasm of the oocyte. We have previously identified hormonally-regulated ascorbic acid transporters in rat granulosa and luteal cells, and herein present preliminary evidence for the presence of a transporter for ascorbic acid in human granulosa–lutein cells. Granulosa–lutein cells were obtained from the follicular fluid of patients undergoing in-vitro fertilization. Following an overnight incubation, the cells were incubated with [14C]-ascorbic acid (0.15 µCi; 150 µM) and ascorbic acid uptake was determined. The uptake of ascorbic acid was saturable with a Michaelis’ constant (Km) and maximum velocity (Vmax) of 21 µM and 3 pmol/10⁶ cells/min respectively. Ouabain, low Na⁺ medium, and dinitrophenol significantly inhibited ascorbic acid uptake (P < 0.05). Neither the presence of insulin, human chorionic gonadotrophin (HCG), insulin-like growth factor (IGF)-I, nor IGF-II affected the uptake of ascorbic acid in a statistically significant fashion. Following saturation of cellular uptake, the ascorbic acid level was estimated to be 1.04 pmoles/10⁶ cells or ~1 mM, a high concentration similar to that seen in rat luteal cells. Active ascorbic acid transport in human granulosa–lutein cells appears to occur via a Na⁺- and energy-dependent transporter, with high levels of ascorbic acid being accumulated in these cells.

Key words: ascorbic acid transporter/human ovary

Introduction

Antioxidant vitamins are notable constituents of the ovary. In particular, ascorbic acid is a water soluble and pre-eminent scavenger of oxygen radicals and serves as an electron donor. In animals, it has been shown that ascorbic acid is concentrated in the granulosa and theca cells of the follicle, as well as the luteal cells of the corpus luteum and the peripheral cytoplasm of the oocyte (Hoch-Ligeti and Bourne, 1948; Deane, 1952). Ascorbic acid is produced by hepatic synthesis in species such as rodents and domestic animals (Grollman and Lehninger, 1957; Levine and Morita, 1985); however, it must be available in the diet of primates and guinea pigs. The vitamin is then transported from blood into the peripheral tissues and accumulated via membrane transporters against a large concentration gradient, most notably in the adrenal glands and the gonads, which contain high concentrations of ascorbic acid (Levine and Morita, 1985; Levine, 1986; Rose, 1988).

Tissue concentrations of ascorbic acid can be rapidly depleted in response to hormones such as luteinizing hormone (LH), prostaglandin (PG)F₂α or PGF₂α, and this response is the basis for a bioassay for some of these hormones (Sayers et al., 1948; Parlow, 1972; Sato et al., 1974; Aten et al., 1992; Endo et al., 1993). Work from our laboratory (Musicki et al., 1996) has demonstrated that ascorbic acid depletion by LH in isolated luteal cells occurred via an increase in ascorbic acid secretion, and that in-vivo depletion in the corpus luteum was blocked by an inhibitor of steroidogenesis. PGF₂α-induced ascorbic acid depletion results from an inhibition of ascorbic acid uptake into the cell, by stimulating cellular secretion, indicating endocrine control of ascorbic acid concentrations in luteal cells and the corpus luteum. Structural involution of the rat corpus luteum is associated with a prolonged depletion of ascorbic acid in association with induction of matrix-degrading enzymes (Sato et al., 1974).

Similarly, the uptake of ascorbic acid in rat granulosa cells was found to be hormonally regulated (Behrman et al., 1996). Our work, together with previously reported findings on the inhibition of apoptosis in granulosa cells by ascorbic acid and other antioxidants (Tilly and Tilly, 1995), suggest that ascorbic acid may play a vital role in the selection of the dominant follicle and in the prevention of atresia or apoptosis (Kramer et al., 1933; Tilly and Tilly, 1995; Tilly et al., 1995; Behrman et al., 1996). No data, however, are available on the presence of such transport mechanisms for ascorbic acid in the human ovary, or on the total accumulation of ascorbic acid in the human ovarian cell. The aim of the present studies was, therefore, to examine ascorbic acid transport in human granulosa–lutein cells and to determine whether such transport is a constitutive or a hormonally-regulated process in human ovarian cells.
Materials and methods

Reagents

Human insulin-like growth factors (IGF)-I and IGF-II were acquired from Collaborative Biomedical Products (Bedford, MA, USA) and Sigma Chemical Company (St Louis, MO, USA) respectively. Insulin was also purchased from Sigma, whereas human chorionic gonadotrophin (HCG) was obtained from Ayerst Laboratories (Rouses Point, NY, USA). Penicillin, streptomycin, and fetal calf serum (FCS) were acquired from Gibco (Grand Island, NY, USA), and all other chemical reagents were obtained from Sigma. L-[1-14C]-ascorbic acid was purchased from Amersham (Arlington Heights, IL, USA), and the lyophilized trace was dissolved in 10 mM sodium phosphate buffer, pH 5.0 and stored under argon at –20°C.

Acquisition and culture of human granulosa–lutein cells.

Granulosa–lutein cells were obtained from the follicular fluid of patients undergoing in-vitro fertilization (IVF). The cells were suspended in minimal essential medium (MEM) 1380 (Gibco) media containing 0.1% bovine serum albumin (BSA), layered onto a Percoll gradient and centrifuged for 30 min. The cells were then collected from the interface and resuspended in media. CD450 magnetic beads (Immunotech, ME, USA) were then used to remove the macrophages and leukocytes (Best et al., 1994). Afterwards, the cells were counted and plated in Dulbecco’s minimal essential medium (DMEM)/F12 (Gibco) containing 1% FCS, at 10^6 cells/well in the presence of 100 IU/ml penicillin and 100 µg/ml streptomycin. The cells were incubated overnight at 37°C, and ascorbic acid uptake was then measured.

Ascorbic acid uptake

Prior to the measurement of ascorbic acid uptake, wells were washed once with Hanks’ balanced salt solution (HBSS), pH 7.4, supplemented with 0.5 mM thiorube and 0.1% BSA. Thiourea was used to stabilize ascorbic acid and thus prevent its oxidation to dehydroascorbic acid. All uptake experiments were carried out in this medium to which [14C]-ascorbic acid (0.15 µCi; specific activity: 6.6–16.8 µCi/µmole) was added. Total ascorbate concentration varied from 30–3000 µM and was adjusted by the addition of unlabelled ascorbate. At the end of the uptake period, which ranged from 15–75 min, as indicated in the text, the culture dish was placed on ice, and the cells were washed three times with 0.3 M sucrose, as previously described (Musicki et al., 1996). The cells were subsequently solubilized in 1 N NaOH for 30 min at 85°C and then neutralized with 1 N HCl prior to assessment of intracellular radioactivity by liquid scintillation counting. Non-specific background radioactivity was determined in identically treated, but non-incubated, cells.

Uptake regulation studies

In the kinetic studies, granulosa cells were incubated overnight in the absence of hormones. The cells were then exposed either to dinitrophenol (1 mM) or low salt media for 10 min, or to 100 µM ouabain for 75 min. Additional experiments were performed in which the cells were preincubated for 48 h with insulin (10 nM), IGF-I or IGF-II (50 ng/well), or HCG (1 IU/ml). The cells were then incubated with [14C]-ascorbic acid (0.15 µCi; 150 µM) for 45 min. Intracellular radioactivity was determined after washing the cells, and ascorbic acid uptake was calculated as described above.

Viability analysis

Cell viability was determined by exclusion of Trypan Blue (Philips, 1973). After isolation of granulosa–lutein cells and enrichment as described above, viability was ~85%.

Figure 1. Time course of ascorbic acid uptake in human granulosa–lutein cells. The mean ± SEM of three replicate experiments are shown. Granulosa–lutein cells were cultured for 24 h with no hormone treatment. Uptake was determined as described the text.

Statistical analysis

Granulosa–lutein cells were pooled from several patients, and equal aliquots were exposed to each treatment in triplicate. Each experiment was repeated at least three times unless otherwise stated in the text. Statistical significance between treatments in the three replicate experiments was determined by one-way analysis of variance. P < 0.05 was considered to be statistically significant.

Results

After a 24 h preincubation period, human granulosa–lutein cells accumulated [14C]-labelled ascorbic acid in a time- and cell-dependent manner (Figure 1). Uptake was linear for 1 h following the addition of ascorbate, and a period of 45 min was chosen for further uptake experiments. Total accumulation of ascorbic acid over a 3 h incubation period in human granulosa–lutein cells was >1000 pmol/10^6 or ~1 mM, a magnitude similar to that seen in rat luteal cells (Musicki et al., 1996). Figure 2 shows that ascorbic acid uptake in cultured human granulosa–lutein cells was a substrate-dependent and saturable process with a Michaeli’s constant (Km) of 21 µM and a maximum velocity (Vmax) of 3 pmol/10^6 cells/min.

Ascorbic acid uptake was noted to be energy-dependent, as dinitrophenol (DNP), an inhibitor of mitochondrial proton transport, or incubation at 4°C severely inhibited ascorbic acid uptake (Figure 3). Ouabain (100 µM), an inhibitor of sodium/potassium ATPase, also significantly inhibited ascorbic acid uptake (P < 0.05); cytochalasin B, an agent that disrupts microfilaments, had no significant effect (Figure 3). These results indicate that the uptake of ascorbic acid by human granulosa–lutein cells involves an active transport process that depends on a sodium gradient established by the sodium/potassium ATPase system, and also requires an intact cytoskeletal system.

Insulin (10 nM) and HCG (1 IU/ml) did not show a statistically significant increase in ascorbic acid uptake when compared with controls. IGF-I (50 ng/well) and IGF-II (50 ng/well) showed no significant effect on ascorbic uptake in human granulosa–lutein cells (Table I).
Ascorbic acid transporter in human granulosa–lutein cells

Figure 2. Michaeli–Menton kinetics of ascorbic acid uptake in human granulosa–lutein cells. The mean ± SEM of three independent experiments are shown (see text for details).

Figure 3. Effect of inhibitors of sodium transport and energy metabolism on ascorbic acid uptake by human granulosa–lutein cells. Reduction of the transcellular Na\(^+\) gradient or uncoupling of oxidative phosphorylation inhibits ascorbic acid uptake in granulosa cells. The mean ± SEM of three independent experiments are shown.

Table I. Agents without effect on ascorbic acid uptake in human granulosa–lutein cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage of control</th>
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<tbody>
<tr>
<td>Control</td>
<td>100 ± 16.4</td>
</tr>
<tr>
<td>HCG</td>
<td>157.5 ± 72.1</td>
</tr>
<tr>
<td>Insulin</td>
<td>156.4 ± 31.0</td>
</tr>
<tr>
<td>IGF-I</td>
<td>117 ± 5.2</td>
</tr>
<tr>
<td>IGF-II</td>
<td>116.3 ± 5.1</td>
</tr>
</tbody>
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HCG = human chorionic gonadotrophin; IGF = insulin-like growth factor.

Discussion

Reactive oxygen species (ROS) such as superoxide anion (O\(_2^-\)) and hydrogen peroxide (H\(_2\)O\(_2\)) are generated under a variety of biological conditions, most notably by phagocytic leukocytes, endothelial cells, and fibroblasts. Ovulation and luteal regression are marked by leukocytic infiltration, which is known to result in superoxide generation in quantities sufficient to cause cell injury and death. Protection against ROS is provided by scavenging ovarian antioxidants, most notably ascorbic acid, which serves as an electron donor in the scavenging of oxygen radicals and in the regeneration of other vital oxidized agents, e.g. vitamin E and glutathione (GSH) (Buettner, 1993; Frei, 1994; Meister, 1994).

A gradient of 20–100-fold exists between extracellular (0.05 mM) and intracellular (1–5 mM) concentrations of ascorbic acid in tissues, e.g. ovary, adrenal, brain, and leukocytes, that contain very high values of this vitamin (Levine and Morita, 1985). Such a concentration gradient implicates that membrane transporters are necessary for the cellular accumulation of ascorbic acid (Rose, 1988) as de-novo synthesis occurs in the liver only. The cellular concentration of ascorbic acid in the human ovary is not known as ascorbic acid is rapidly depleted by the mere isolation of tissue (Behrman, 1996; Musicki et al., 1996). Although the ascorbic acid transporter has not been cloned, we have identified an energy and Na\(^+\)-dependent ascorbic acid transporter in rat luteal and, more recently, rat granulosa cells (Behrman et al., 1996; Musicki et al., 1996).

In the present study, we demonstrated that human granulosa–lutein cells accumulated ascorbic acid in a time- and cell-dependent manner. Uptake was a substrate-dependent and a saturable process, with a K\(_m\) of 21 µM and a V\(_{max}\) of 3 pmol/10\(^6\) cells/min respectively. As such, the human granulosa–lutein cell ascorbic acid transport system showed characteristics similar to those of transporters in other cells (Moger, 1987; Rose, 1988; Musicki et al., 1996).

Ascorbic acid uptake in human granulosa–lutein cells was also found to be driven by Na\(^+\)- and energy-dependent transporters, as ouabain an inhibitor of sodium/potassium ATPase, and incubation of granulosa–lutein cells in a low Na\(^+\) medium significantly inhibited ascorbic acid uptake. Similarly, DNP,
an inhibitor of mitochondrial proton transport, significantly inhibited ascorbic acid transport, which suggests that the transport of ascorbic acid by human granulosa-lutein cells involves an active process that depends on a sodium gradient established by the Na\(^+\)/K\(^+\) ATPase system. However, ascorbate uptake was unaffected by HCG, insulin, IGF-I, or IGF-II perhaps due to prior stimulation of these granulosa-lutein cells at the time of IVF.

Ascorbic acid plays a vital role in follicular development as scorbutic guinea pigs were shown to be anovulatory with marked follicular degeneration and these animals also had increased rates of implantation failure, and increased spontaneous abortions (Kramer et al., 1933). More recent studies have shown that ascorbic acid blocks apoptosis in cultured follicles, which led Tilly and Tilly (1995) to the conclusion that ascorbic acid and other antioxidants serve a role to prevent atresia. More recent evidence from our laboratory showed that ascorbic acid transport is up-regulated by follicle stimulating hormone (FSH) and IGF-I (Behrman et al., 1996), and that antioxidants block the spontaneous resumption of meiosis in rat oocytes removed from the follicular environment (M.Guarnacia et al., manuscript in preparation).

Interestingly, ascorbic acid supplementation increased the ovulation inducing effects of clomiphene in anovulatory women by an apparent local ovarian effect (Igarashi, 1997). Beyond these studies, little information is available on the role of ascorbic acid in human ovarian function although it has been recently speculated to be a fertility enhancing factor (Millar, 1992; Luck et al., 1995). In this study, we have demonstrated the presence of an active ascorbic acid transport mechanism in ovarian granulosa–luteal cells, which paves the way for further studies on the role of ascorbic acid in follicular development, ovulation, and luteal function in the human ovary.

Acknowledgements

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References


