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17β-estradiol inhibition of ascorbic acid accumulation in human intestinal Caco-2 cells

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Abstract

We investigated the effect of estrogen on the accumulation of ascorbic acid by human intestinal Caco-2 cells. 17β-estradiol, synthetic estrogen diethylstilbestrol, and partial agonist tamoxifen were found to inhibit ascorbic acid accumulation in a dose-dependent fashion. The inhibitory effect of estrogens can be observed at as short as 5 min of incubation. An additive effect was observed when they were used in combination. Similar to dietary flavonoids, inhibition was also observed in two other intestinal cell lines, HT-29 and IEC-6. These chemicals affected both Na^+-dependent and Na^+-independent(K^+ substituting Na^+) accumulation of ascorbic acid and did not affect the efflux of accumulated ascorbic acid. Kinetic analysis of diethylstilbestrol showed a non-competitive inhibition with an apparent K_i of 23 μM. The hormone-ascorbic acid interaction in the intestinal cell could help to explain the known reduction in blood ascorbic acid level among oral contraceptive users and female guinea pigs given contraceptive hormones.

Keywords: Ascorbic acid; Estrogen; Diethylstilbestrol; Tamoxifen; Flavonoid; Caco-2 cell

1. Introduction

Ascorbic acid (reduced form of vitamin C) functions in cells as an antioxidant (Beyer, 1994), enzyme cofactor (Padh, 1990) and growth modulator (Alcain and Buron, 1994). It is synthesized endogenously in liver by most species with the exception of human, guinea pigs and primates (Burns, 1957). Intestinal handling of ascorbic acid in human thus represents a critical step in the acquiring of ascorbic acid from dietary sources. Previous studies using intestinal preparations from human (Stevenson, 1974) and guinea pigs (Mellors et al., 1977; Siliprandi et al., 1979; Bianchi et al., 1986) have shown that ascorbic acid is transported through the luminal membrane of intestine by a Na^+-dependent carrier-mediated pathway. Similar Na^+-dependent transport of ascorbic acid was also demonstrated in an alternative model of human intestinal cells, Caco-2 cells (Kuo et al., 1997). Inside the cells, ascorbic acid is accumulated against a concentration gradient (Stevenson, 1974; Mellors et al., 1977; Bianchi et al., 1986; Kuo et al., 1997).

While the regulation of ascorbic acid transport has been studied in neutrophils (Washko and Levine, 1992), ciliary epithelium (Delamere et al., 1993), and astrocytes (Siushansian and Wilson, 1995), little is known about the regulation of cellular accumulation of ascorbic acid in the intestinal cells. Changes in the handling of ascorbic acid by intestinal cells could affect ascorbic acid absorption. In our previous studies, we found that the accumulation was decreased by dietary components, quercetin (a flavonoid) and genistein (an isoflavonoid) (Kuo et al., 1997). This report represents our attempt to further understand the regulation of ascorbic acid accumulation in human intestinal cells.

We hypothesized that other chemicals similar to flavonoids will also affect the accumulation of ascorbic acid. One potential candidate is estrogen. There are limited similarities between the structure of flavonoids/isoflavonoids and that of 17β-estradiol. Propertywise, genistein was found to stimulate the uterine growth of female rats similar to estrogen (Santell et al., 1997). In vitro, flavonoids and isoflavonoids were shown to interact with estrogen receptor (Miksicek, 1993; Makela et al., 1994)

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and type II estrogen binding sites (Scambia et al., 1991; Ranelletti et al., 1992; Markaverich and Gregory, 1993; Piantelli et al., 1995; Caltagirone et al., 1997). Although the mechanism that mediates the inhibitory effect of flavonoids on ascorbic acid accumulation is not clear, the inhibition may also be observed in cells treated with 17β-estradiol or other synthetic estrogens.

There are also evidences supporting an interaction between estrogen and ascorbic acid under physiological condition. Oral contraceptive users were found to have a lower level of plasma ascorbic acid compared to control women of similar age and health condition. Their ascorbic acid levels in leukocytes and platelets were also lower (Briggs and Briggs, 1972; Rivers and Devine, 1972; McLeroy and Schendel, 1973; Rivers, 1975). The effect could be observed at two months after the initiation of oral contraceptive usage (Briggs and Briggs, 1972). Physiological compensation was not noted as the effect remained after 3 or more years of usage (McLeroy and Schendel, 1973). Female guinea pigs given contraceptive hormones were also found to have a lower level of ascorbic acid in blood parameters compared to the control animals (Basu, 1986). A decrease in the intestinal ascorbic absorption upon oral contraceptive usage was one of the many mechanisms proposed (McLeroy and Schendel, 1973) and urinary ascorbic acid excretion was found to be lower in oral contraceptive users after a bolus dose (Rivers and Devine, 1972). Our in vitro study addressing the effect of estrogen on ascorbic acid accumulation by intestinal cells may help to understand the drug-nutrient interaction for the hormone users.

2. Materials and methods

2.1. Materials

Quercetin was purchased from Fluka Chemie (Switzerland). L-Ascorbic acid, 17β-estradiol, diethylstilbestrol, phenol reagent and tamoxifen were purchased from Sigma (St. Louis, MO). Genistein was from Calbiochem (La Jolla, CA). The reagents for cell culture were all purchased from Gibco BRL (Gaithersburg, MD) except for fetal bovine serum (HyClone Laboratories, Logan, UT). L-[Carboxyl^{14}C]ascorbic acid (specific activity 14.3 mCi/mmol) was purchased from Amersham (Buckinghamshire, England). To prevent oxidation, [^{14}C]ascorbic acid was dissolved in 100% ethanol, aliquoted to 2.5 μCi per vial for drying under nitrogen, and then stored at −70°C until use. Quercetin, genistein and estrogens were dissolved in 100% ethanol as stock solutions and used for treatment. Ethanol was added to the control wells during the experiment at the same concentration as in the treated groups. Ethanol at the concentration used (0.4%–1%) did not affect the cellular accumulation of ascorbic acid.

2.2. Cell culture

Human colon adenocarcinoma cells, Caco-2 (Fogh et al., 1977), were originally purchased from American Type Culture Collection and were used between passage 24 to 43. They were cultured in high glucose Dulbecco's modified Eagle medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.1 mM non-essential amino acid as reported before (Kuo, 1996). Another line of human colon

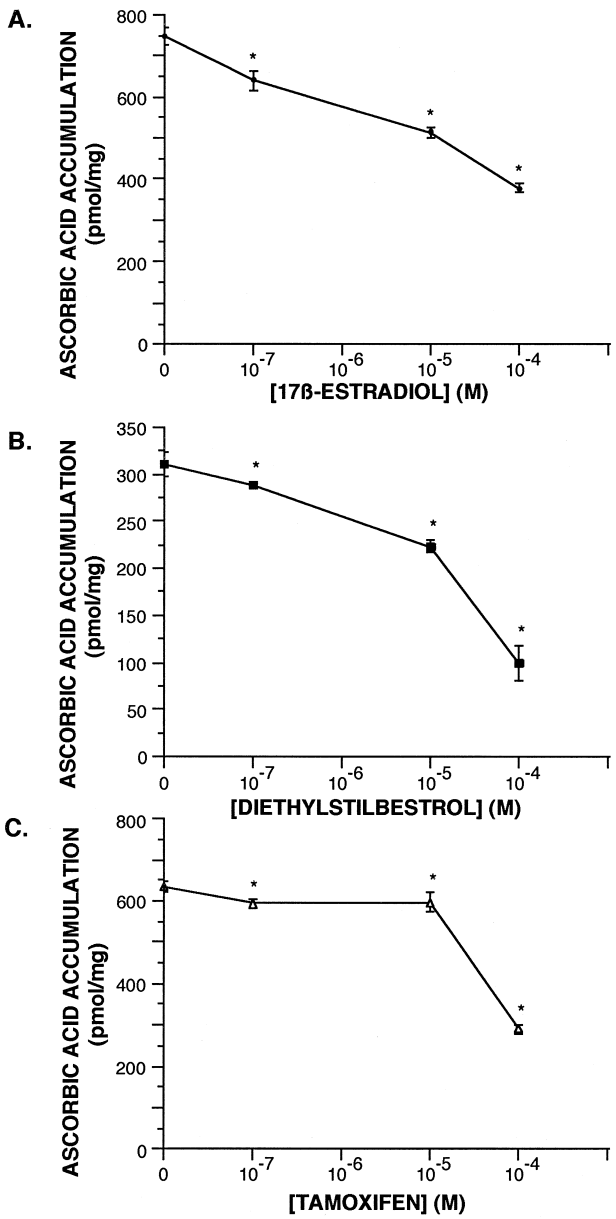

Fig. 1. Dose-dependent inhibition of ascorbic acid accumulation by 17β-estradiol and synthetic estrogens in Caco-2 cells. Accumulation was measured after 30 min incubation with 10 μM [^{14}C]ascorbic acid in the absence or presence of various concentrations of (A) 17β-estradiol; (B) diethylstilbestrol; (C) tamoxifen. Each point represents the mean ± S.D. of three separate wells.

Table 1
Effect of 17β-estradiol and diethylstilbestrol on ascorbic acid accumulation in human intestinal HT-29 cells and rat intestinal IEC-6 cells

Treatment	Ascorbic acid accumulation (pmol/mg protein)	
	HT-29 cells	IEC-6 cells
Control	202 ± 16	386 ± 41
17β-Estradiol	91 ± 13[a]	199 ± 32[a]
Diethylstilbestrol	33 ± 2[a]	33 ± 4[a]

Cells were incubated with 11 μM [^{14}C]ascorbic acid and 100 μM inhibitor for 30 min and then cellular accumulation of [^{14}C]ascorbic acid was measured. Data shown are the mean ± S.D. of three separate wells.
[a] Indicates significant differences at $P < 0.05$ by post-hoc Dunnett's test compared to the respective control.

adenocarcinoma cells, HT-29, and rat intestinal non-transformed crypt cells, IEC-6, were cultured as described before (Kuo et al., 1997). Cells at the density of 1.5×10^4–2×10^4 cells/cm^2 were seeded in six-well culture plate for experiment. Growth medium was changed every other day. All experiment were performed at 6 days post-seeding when monolayers were just confluent.

2.3. [^{14}C]ascorbic acid accumulation study

Immediately before each experiment, cell monolayers were rinsed twice and then incubated with 2 ml warm buffer solution containing Hank's balanced salt solution, 25 mM HEPES, 100 units/ml penicillin and 100 μg/ml streptomycin at pH 7.4 (HBSS). [^{14}C]ascorbic acid solution was prepared by mixing freshly prepared cold ascorbic acid with freshly reconstituted L-[carboxyl-^{14}C]ascorbic acid stock solution to give a final concentration of 10 μM and a final radioactivity of around 0.05 μCi per well. [^{14}C]ascorbic acid transport study was performed as described before (Kuo et al., 1997). After the termination of the experiment, the cells were lysed in 3 ml of 0.1% SDS solution overnight at 4°C. Complete cell lysate was prepared the next day by sonication after 1 h low-speed mixing on a platform mixer at room temperature.

In the experiments where Na^+-free condition was required, Na^+ ion in HBSS was iso-osmotically replaced by K^+ ion. In the efflux experiments, cells were preloaded with 10 μM [^{14}C]ascorbic acid for 30 min at 37°C. [^{14}C]ascorbic acid solution was removed at the end of the preloading. The cell monolayers were rinsed with warm Hank's solution for three times. They were then incubated with 2 ml of Hank's buffer solution containing 10 μM freshly prepared ascorbic acid in the presence or absence of the compound tested to allow for 30-min efflux. The addition of cold ascorbic acid in the efflux medium was to mimic the condition when net accumulation was measured.

Aliquots of cell lysate were transferred to scintillation vials and mixed with 3 ml of Ecolite (+) scintillation fluid (ICN Biomedicals, Irvine, CA). The radioactivity was then measured by a liquid scintillation counter (Beckman, Model LS 1801). The amount of protein in the cell lysate was determined by a modified Lowry method (Peterson, 1983) using bovine serum albumin as the standard.

3. Results

3.1. The effect of 17β-estradiol and synthetic estrogens

In our previous study (Kuo et al., 1997), several flavonoids were found to decrease the net accumulation of ascorbic acid in Caco-2 cells. As shown in Fig. 1, 17β-estradiol, diethylstilbestrol and tamoxifen all significantly decreased ascorbic acid accumulation in Caco-2 cells. The effects of 17β-estradiol, diethylstilbestrol and tamoxifen

Table 2
Effect of combination of 17β-estradiol, diethylstilbestrol and tamoxifen on ascorbic acid accumulation in Caco-2 cells

Treatment	Drug concentration (μM)	Ascorbic acid accumulation (pmol/mg)
Control	–	566 ± 10
17β-estradiol	20	434 ± 23
Diethylstilbestrol	20	332 ± 22
Tamoxifen	20	547 ± 3
17β-estradiol + Diethylstilbestrol	20/20	277 ± 19
Diethylstilbestrol + Tamoxifen	20/20	307 ± 14
17β-estradiol + Tamoxifen	20/20	412 ± 17
17β-estradiol + Diethylstilbestrol + Tamoxifen	20/20/20	246 ± 6

Caco-2 cells were incubated with 10 μM [^{14}C]ascorbic acid and inhibitor(s) for 30 min and then cellular accumulation of [^{14}C]ascorbic acid was measured. All the accumulation studies were carried out on the sixth day after seeding. Data shown are the mean ± S.D. of three separate wells.

were dose-dependent. Across the concentration range we tested, diethylstilbestrol was the most potent inhibitor, followed by 17β-estradiol and then tamoxifen.

We have shown previously that quercetin and genistein also significantly inhibited ascorbic acid accumulation in two other intestinal cell lines, human HT-29 and rat IEC-6 cells (Kuo et al., 1997). In this study, 17β-estradiol and diethylstilbestrol were found to inhibit the accumulation of ascorbic acid in these two lines of intestinal cells as well (Table 1). In these two types of cells, diethylstilbestrol was also a more potent inhibitor compared to 17β-estradiol.

The effects of 17β-estradiol, diethylstilbestrol and tamoxifen were found to be additive at 20 μM (Table 2). Although the effect of tamoxifen was very limited by itself at 20 μM, it furthered the inhibition when it was combined with 17β-estradiol or diethylstilbestrol. When all three forms of estrogens were added together, the accumulation of ascorbic acid was decreased to less than half (43%) of the control.

3.2. Time course of inhibition by 17β-estradiol and synthetic estrogens

In our study of flavonoids, it was found that the inhibitory effect of flavonoids on ascorbic acid accumulation can be observed in as short as 10 min incubation (Kuo et al., 1997). In Fig. 2, 17β-estradiol and diethylstilbestrol were able to inhibit ascorbic acid accumulation within 5 min of incubation. For both compounds at the concentrations used, the levels of inhibition were similar from 5 min to 30 min of incubation.

3.3. The role of Na^+-dependent ascorbic acid transporter

Na^+-dependent ascorbic acid transport has been demonstrated in Caco-2 cells (Kuo et al., 1997). On possible mechanism that 17β-estradiol and related compounds can affect the accumulation of ascorbic acid is through blocking the Na^+-dependent transport system. As shown in

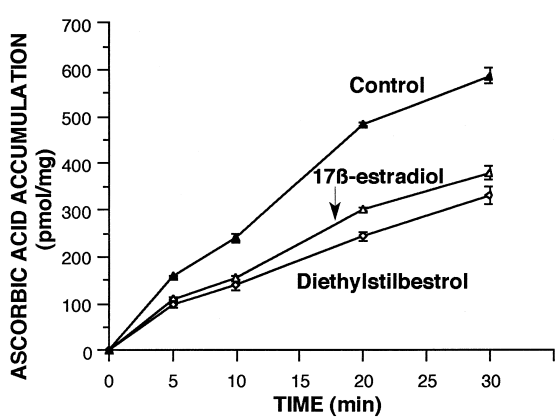

Fig. 2. Time course of inhibition of ascorbic acid accumulation by estrogens. Caco-2 cells were incubated with 10 μM [^{14}C]ascorbic acid in the absence or presence of 50 μM 17β-estradiol or 20 μM diethylstilbestrol. Each point represents the mean ± S.D. of three separate wells at one time point.

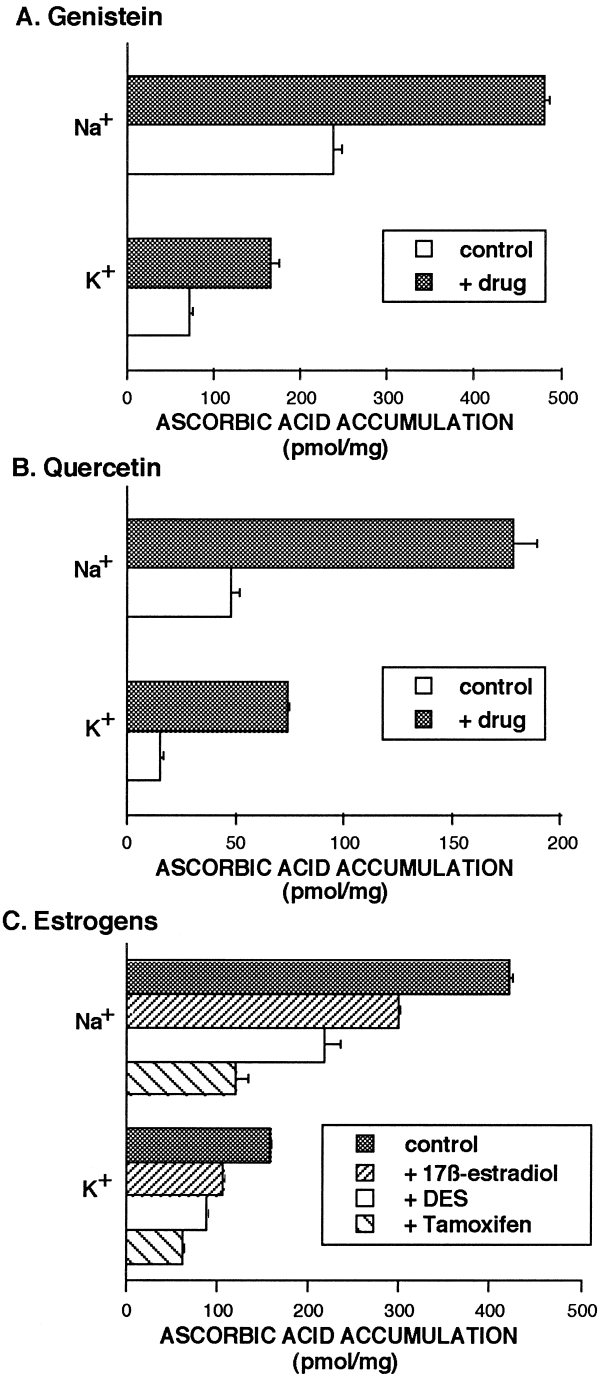

Fig. 3. Effect of flavonoids and estrogens on Na^+-dependent and Na^+-independent ascorbic acid accumulation in Caco-2 cells. Accumulation was measured after (A) 30 min incubation with 10 μM [^{14}C]ascorbic acid in the absence of presence of 100 μM genistein; (B) 20 min incubation with 6 μM [^{14}C]ascorbic acid in the absence of presence of 50 μM quercetin; (C) 30 min incubation with 10 μM [^{14}C]ascorbic acid in the absence of presence of 50 μM 17β-estradiol, 20 μM diethylstilbestrol or 50 μM tamoxifen. The transport solution contained regular HBSS (Na^+) or HBSS with K^+ substituting for Na^+ (K^+). Each bar represents the mean ± S.D. of three separate wells.

Fig. 3A, when Na^+ ion was substituted with K^+ ion, ascorbic acid accumulation was decreased to $34 \pm 3\%$ of the control. Genistein at 100 μM inhibited ascorbic acid accumulation in the Na^+-containing and Na^+-free (K^+-containing) solutions similarly when compared to their respective controls ($50 \pm 2\%$ in the presence of Na^+ versus $44 \pm 2\%$ in the presence of K^+). Quercetin was also found to inhibit ascorbic acid accumulation independent of Na^+ status as shown in Fig. 3B. Quercetin at 50 μM reduced cellular ascorbic acid accumulation to $27 \pm 2\%$ during a 20 min incubation in the presence of Na^+. Using K^+ but otherwise identical condition, quercetin decreased cellular ascorbic acid accumulation to $21 \pm 4\%$ of its control. As shown in Fig. 3C, 17β-estradiol, diethylstilbestrol and tamoxifen apparently inhibited both Na^+-dependent and Na^+-independent ascorbic acid transport as well. The degree of inhibition by each compound, again, was similar under the Na^+ and K^+ conditions.

3.4. Efflux of ascorbic acid in the presence of inhibitors

Changes in the efflux of ascorbic acid could also account for the inhibitory effect of various chemicals. The efflux of ascorbic acid was measured from cells that were preloaded with [^{14}C]ascorbic acid for 30 min. The efflux of ascorbic acid progressed very slowly. After 30 min of efflux, only $5.3 \pm 0.6\%$ of the total cellular accumulation was released into the incubation solution in control group (Table 3). When quercetin or genistein was included in the efflux medium, [^{14}C]ascorbic acid efflux was not significantly altered (6.3 ± 1.1 and $6.7 \pm 0.6\%$ of the total accumulation, respectively). In a separate experiment, the efflux of ascorbic acid from preloaded cells was measured in the presence and absence of 100 μM diethylstilbestrol. The 30 min efflux accounted for $10 \pm 2\%$ and $11 \pm 3\%$ of the loaded ascorbic acid in control group and in the diethylstilbestrol group, respectively.

Table 3
Effect of flavonoids on the efflux of ascorbic acid from Caco-2 cells

Ascorbic acid (pmol/mg protein)	Control	Quercetin	Genistein
Total uptake	526 ± 32	554 ± 52	539 ± 54
Cell-associated	498 ± 28	519 ± 53	503 ± 50
Efflux	28 ± 5	34 ± 6	36 ± 5
Efflux (%)	(5.3 ± 0.6)	(6.3 ± 1.1)	(6.7 ± 0.6)

Caco-2 cells were loaded with 11 μM [^{14}C]ascorbic acid for 30 min, and then allowed to efflux for 30 min in the presence of 10 μM unlabeled ascorbic acid and 100 μM flavonoid in a fresh transport buffer. The study was carried out on the sixth day after cell seeding. [^{14}C]ascorbic acid in the cells (Cell-associated) and in the transport buffer (Efflux) were measured at the end of 30-min efflux period and the sum of Cell-associated and Efflux represented the total [^{14}C]ascorbic acid uptake during the loading period. The extent of efflux expressed as percentage of the total [^{14}C]ascorbic acid uptake is given in parentheses. There are no significant differences in percentages of efflux among these three groups by ANOVA. Each value is the mean ± S.D. of three separate wells.

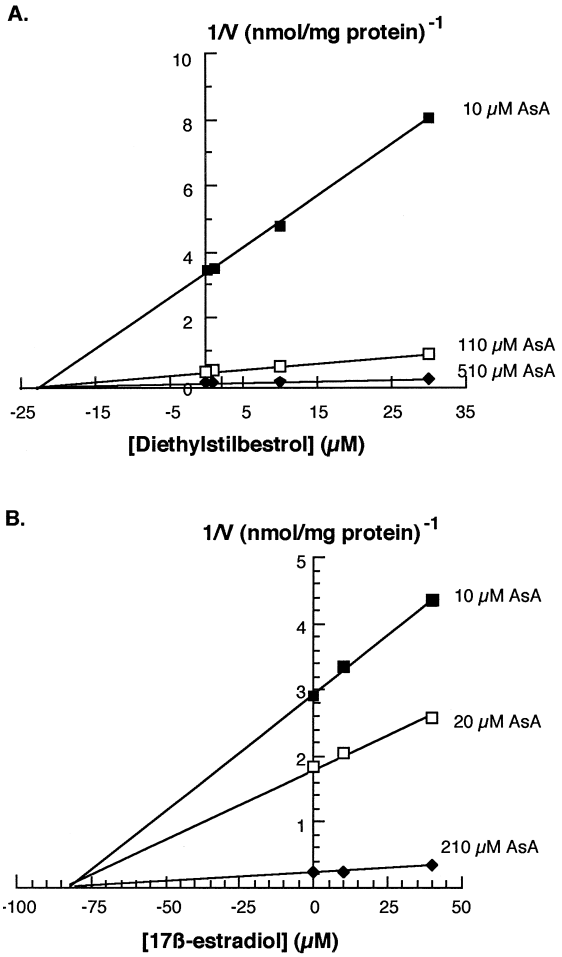

Fig. 4. Dixon plot of diethylstibestrol and 17β-estradiol inhibition of ascorbic acid accumulation in Caco-2 cells. Accumulation was measured after 15 min incubation with different concentrations of ascorbic acid and inhibitors. (A) 10, 110 or 510 μM [^{14}C]ascorbic acid in the absence or presence of various concentrations of diethylstilbestrol; (B) 10, 20 or 210 μM [^{14}C]ascorbic acid in the absence or presence of various concentrations of 17β-estradiol. Each point represents the mean of three wells.

3.5. Kinetics of inhibition

In Fig. 4, we used Dixon plot to determine the mode of inhibition of 17β-estradiol and diethylstilbestrol. The results indicated that both 17β-estradiol and diethylstilbestrol are non-competitive inhibitor of ascorbic acid with an apparent K_i of 82 μM and 23 μM, respectively. This result is consistent with the notion that diethylstilbestrol is a more potent inhibitor of ascorbic acid accumulation than 17β-estradiol.

4. Discussion

We have previously observed that flavonoids inhibited ascorbic acid accumulation in human intestinal Caco-2 cells (Kuo et al., 1997). Because of the known estrogenic

effect of flavonoids (see Section 1), we hypothesized that estrogens would inhibit the accumulation of ascorbic acid similar to flavonoids. Based on our studies, 17β-estradiol and its agonists do inhibit the accumulation of ascorbic acid by intestinal cells. Diethylstilbestrol is the most potent inhibitor followed by 17β-estradiol and then tamoxifen (Figs. 1, 2 and 4 and Table 2). The observation that estrogen inhibits ascorbic acid accumulation in the intestinal cells maybe the underlying cause of poor ascorbic acid status in oral contraceptive users (Briggs and Briggs, 1972; McLeroy and Schendel, 1973; Rivers, 1975). Understanding the mechanism behind the inhibition may help to prevent or alleviate the adverse effect.

It is unlikely that the effects of these estrogens were mediated through known estrogen receptors. Estrogen receptors were known to modulate biological events through the genomic effect (Gronemeyer, 1991). The inhibitory effect in this study, on the other hand, was observed upon short duration of incubation (as short as 5 min) (Fig. 2). It is thus unlikely that the inhibition was a result of gene suppression. The K_i we observed for estrogens was also not consistent with an event mediated by estrogen receptors. The concentration of 17β-estradiol and diethylstilbestrol required to activate estrogen receptor is usually around 10^{-9} M (Kuiper et al., 1996). In our experiments, the K_i for both diethylstilbestrol and 17β-estradiol were greater than 10^{-5} M (Fig. 4) and very little inhibition was observed at 10^{-7} M (Fig. 1).

We have analyzed the effect of these chemicals on different elements that were involved in the net cellular ascorbic acid accumulation. The influx of ascorbic acid into Caco-2 cells is known to be mediated mainly by Na^+-dependent process although Na^+-independent influx also occurs (Kuo et al., 1997). In comparing Na^+-dependent and Na^+-independent transport of ascorbic acid, it was found that these two pathways were affected similarly by flavonoids and estrogens (Fig. 3). The exact nature of the Na^+-independent influx of ascorbic acid is not clear and we cannot rule out the presence of voltage-sensitive ascorbic acid influx under K^+-replacement condition. Nonetheless, previous study using guinea pig intestinal brush border membrane vesicles concluded that the sodium-dependent transport of ascorbic acid was electroneutral (Siliprandi et al., 1979).

The effect of estrogen was also not a consequence of changes in the efflux of ascorbic acid. The efflux of preloaded [^{14}C]ascorbic acid was not affected by the flavonoid or diethylstilbestrol treatment (Table 3). In fact, the preloaded radioactive ascorbic acid remained mostly in the cells ($\geq 90\%$) even after 30 min incubation. The slow efflux implies low membrane permeability for the outward movement of ascorbic acid, or a binding of ascorbic acid to the intracellular components that prevents the outward movement of ascorbic acid. Slow efflux of loaded ascorbic acid has been observed in other cell types as well. Efflux of ascorbic acid from primary and second passage of bovine retinal pigment epithelial cells over 20 min was found to be 11 ± 2 and $16 \pm 3\%$, respectively (Khatami, 1988). A lack of changes in ascorbic acid efflux upon treatment is consistent with the results of our [^{14}C]mannitol experiment. Cellular accumulation of membrane-impermeable [^{14}C]mannitol is an indicator for cellular membrane permeability. Similar to flavonoids (Kuo et al., 1997), 17β-estradiol at 100 μM did not affect the accumulation of [^{14}C]mannitol (results not shown) and thus supported that the general permeability of membrane is not affected. Recently, it was suggested that estrogen may interact with membrane receptors and affect cellular cAMP level in enterocytes (Picotto et al., 1996). The effect of cAMP level on ascorbic acid accumulation in intestinal cells is not clear but cAMP level was found to affect ascorbic acid accumulation in human neutraphils (Washko and Levine, 1992), rabbit non-pigmented ciliary epithelium (Delamere et al., 1993), rat astrocytes (Siushansian and Wilson, 1995; Siushansian et al., 1997) and granulosa cells (Behrman et al., 1996). Further study is needed to determine the role of cAMP level changes in the observed inhibition by estrogens and flavonoids.

Ascorbic acid and estrogens have very different structure features. Not surprisingly, the inhibition by diethylstilbestrol and 17β-estradiol on ascorbic acid accumulation was non-competitive (Fig. 4). Although this observation does not provide information on the actual mechanism of inhibition, this mode of inhibition is consistent with the clinical observation. In studies involved oral contraceptive users, it was found that supplementation with ascorbic acid cannot completely reverse the adverse effect due to hormone therapy (McLeroy and Schendel, 1973; Rivers, 1975). Hormone therapy is now available for medical purposes other than contraception. The potential implication of this drug-nutrient interaction in other clinical settings also need to be addressed in future studies.

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