Glucocorticoid Stimulation of Na^+-Dependent Ascorbic Acid Transport in Osteoblast-Like Cells

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Ascorbic acid (AA) is an essential cofactor for osteoblast differentiation both in vivo and in vitro. Before it can function, this vitamin must be transported into cells via a specific Na^+-dependent AA transporter. In this study, we examine the regulation of this transport activity by glucocorticoids, a class of steroid hormones known to stimulate in vitro osteoblast differentiation. Dexamethasone stimulated Na^+-dependent AA transport activity approximately twofold in primary rat calvarial osteoblasts. Effects of hormone on ascorbic acid transport were rapid (detected within 24 h) and were maximally stimulated by 25–50 nM dexamethasone. Similar effects of dexamethasone on transport activity were also observed in murine MC3T3-E1 cells. This preosteoblast cell line was used for a more detailed characterization of the glucocorticoid response. Transport activity was stimulated selectively by glucocorticoids (dexamethasone > corticosterone) relative to other steroid hormones (progesterone and 17-β-estradiol) and was blocked when cells were cultured in the presence of cycloheximide, a protein synthesis inhibitor. Kinetic analysis of AA transporter activity in control and dexamethasone-treated cells indicated a K_m of approximately 17 μM for both groups. In contrast, dexamethasone increased V_{max} by approximately 2.5-fold. Cells also contained an Na^+-independent glucose transport activity that has been reported in other systems to transport vitamin C as oxidized dehydroascorbic acid. In marked contrast to Na^+-dependent AA transport, this activity was inhibited by dexamethasone. Thus, glucocorticoids increase Na^+-dependent AA transport in osteoblasts, possibly via up-regulation of transporter synthesis, and this response can be resolved from actions of glucocorticoids on glucose transport. J. Cell. Physiol. 176:85–91, 1998. © 1998 Wiley-Liss, Inc.

Ascorbic acid (AA; reduced vitamin C) is essential for the formation of bone and other connective tissues and is necessary for the in vivo and in vitro differentiation of osteoblasts (Franceschi, 1992; Togari et al., 1995). Actions of AA on osteoblast differentiation require the synthesis of a collagenous extracellular matrix (ECM) and may be mediated by integrin:matrix interactions (Franceschi, 1992; Franceschi and Iyer, 1992; Franceschi et al., 1994; Takeuchi et al., 1996). We recently showed that AA up-regulates expression of the osteocalcin gene by stimulating activity of the osteoblast-specific transcription factor, OSF2 (Xiao et al., 1997). This factor, which is encoded by the Cbfa1 gene, is essential for osteoblast formation both in vivo and in vitro (Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997). Thus, vitamin C is able to regulate fundamental transcriptional controls necessary for bone formation.

AA must be transported into osteoblasts before it can act. Although both Na^+-dependent (Diliberto et al., 1983; Helbig et al., 1989; Wilson and Dixon, 1989) and Na^+-independent transport of vitamin C has been described in various cell types (Ingermann et al., 1986; Mann and Newton, 1975; Vera et al., 1995), the former process is the predominant route for vitamin uptake by osteoblasts and is essential for the intracellular accumulation of vitamin C and cellular responsiveness (Dixon et al., 1991; Franceschi et al., 1995; Wilson and Dixon, 1989). Although changes in AA transport would be predicted to have profound effects on osteoblast function, only a limited number of studies have examined the regulation of AA transport by exogenous factors. Dixon and coworkers showed that AA pretreatment of UMR-106 cells down-regulates transport activity

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(Dixon and Wilson, 1992a), whereas TGF-β treatment of the same cells increases transport (Dixon and Wilson, 1992b).

Steroid hormones such as, glucocorticoids, progestins, and estrogens, are known to affect osteoblast activity (Bellows et al., 1987, 1990; Gray, 1989; Ishida et al., 1996), but their effects on AA transport have not been previously examined. In the present study, we tested several steroid hormones for their ability to stimulate Na^+-dependent AA transport in primary cultures of rat calvarial osteoblasts and murine MC3T3-E1 preosteoblast cells. Of the hormones tested, as will be shown, only glucocorticoids were able to stimulate AA transport, and this stimulation required de novo protein synthesis.

MATERIALS AND METHODS

Reagents

Tissue culture media and fetal bovine serum were obtained from HyClone Laboratories, Inc. (Logan, UT). Penicillin/streptomycin was purchased from Gibco BRL Life Technologies (Gaithersburg, MD). *L*-[carboxyl-^{14}C]AA (specific activity 16.7 mCi/mmol) and 2-deoxy-*D*-[2,6-^{3}H]glucose (specific activity 57.5 Ci/mmol) were obtained from Amersham Life Science (Arlington Heights, IL). Dexamethasone (Dex), *L*-AA, *DL*-homocysteine, *N*-methyl-*D*-glucamine, and 2-deoxy-*D*-glucose were purchased from Sigma Chemical Company (St. Louis, MO). All other chemicals were of analytical grade.

Cell culture

Primary cultures of rat osteoblast-like cells were established from neonatal rat calvaria by using the procedure of Pockwinse et al. (1992). Briefly, calvaria were dissected from newborn rat pups, isolated from periosteum, and subjected to sequential digestions of 20, 40, and 90 min in collagenase A (2 mg/ml; Boehringer-Mannheim, Indianapolis, IN) and 0.25% trypsin. Cells from the third digest were washed, counted, and plated in AA-free modified Eagle's medium (α-MEM)/10% fetal bovine serum containing 100 U/ml penicillin and streptomycin at a density of 20,000 cells/cm^2. Primary cultures were used without passage.

MC3T3-E1 cells, a generous gift from Dr. M. Kumegawa (Meikai University, Sakado, Japan), were plated at a density of 50,000 cells/cm^2 (unless otherwise specified) and grown in α-MEM containing 10% fetal bovine serum and 1% penicillin/streptomycin. Treatments were commenced after a 24-h attachment period. Hormones were prepared in 1,000× aliquots by using absolute ethanol as the vehicle and diluted in α-MEM at the time of treatment. Control studies indicated that the ethanol vehicle did not affect transport activity. Cells were counted by using a Coulter Counter ZM (Hialeah, FL) or a hemocytometer.

Measurement of ascorbate uptake

Measurement of *L*-[1-^{14}C]AA was conducted essentially according to the method of Wilson and Dixon (1989). Cells were rinsed with serum-free AA transport buffer (which consisted of 134 mM NaCl, 5.4 mM KCl, 1.8 $CaCl_2$, 0.8 mM $MgSO_4$, 10 mM glucose, and 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) adjusted to pH 7.3 with NaOH) and were then incubated in the same buffer containing 2.5 μM *L*-[1-^{14}C]AA at 37°C for 15 min. Previous studies demonstrated that uptake is linear for at least 30 min under these conditions. Cellular accumulation of AA was measured by using a scintillation counter, and the data were normalized to cell number or total cell protein. The same protocol was followed for studying deoxyglucose transport, except that the 10 mM glucose in the AA transport buffer were replaced with 5 mM 2-deoxy-*D*-glucose and 5 mM sucrose. To examine the Na^+ independence of deoxyglucose transport, Na^+ was isosmotically replaced with *N*-methyl-*D*-glucamine. Cells were rinsed with serum-free deoxyglucose transport buffer and were then incubated in the same buffer containing 1 μCi 2-deoxy-*D*-[2,6-^{3}H]glucose/ml of transport buffer to give a final specific activity of 0.2 mCi/mmol. Cellular accumulation of deoxyglucose was measured with a scintillation counter, and data were normalized to cell number.

Statistical analysis

All values are expressed as means ± S.D. of triplicate transport assays. Error bars were omitted from figures when they were smaller than symbols. All experiments were repeated at least twice and produced qualitatively identical results. Dunnett's multiple comparisons test was used to assess statistical significance among treatments. The apparent Michaelis-Menten constant (K_m) and the maximal rate of AA uptake (V_{max}) were calculated by fitting data to idealized Michaelis-Menten kinetics by using a Levenburg-Marquardt algorithm in the Curve-Fitter program of SlideWrite Plus (ver. 3; Advanced Graphics Software, Carlsbad, CA).

RESULTS

Dex stimulation of AA uptake in primary rat calvarial osteoblasts and MC3T3-E1 cells

To begin exploring possible actions of steroid hormones on the Na^+-dependent AA transport activity of osteoblast-like cells, we treated both primary rat calvarial osteoblasts and murine MC3T3-E1 cells with 17-β-estradiol, progesterone, corticosterone, and dex compounds and measured transport activity after 2 days. In this preliminary experiment (results not shown), only Dex and corticosterone exhibited significant activity. Figure 1 shows that Dex stimulated Na^+-dependent AA uptake in primary rat calvarial osteoblasts (Fig. 1A) or MC3T3-E1 cells (Fig. 1B) after as little as 18–24 h. Transport activity continued to increase up to the 48-h time point. During this time interval, Dex did not significantly affect either cell number or protein content. For example, day 2 control MC3T3-E1 cultures contained $1.50 \pm 0.1 \times 10^6$ cells/plate and 0.439 ± 0.09 mg protein, whereas Dex-treated samples contained $1.30 \pm 0.2 \times 10^6$ cells/plate and 0.437 ± 0.08 mg protein. At later times, hormone treatment inhibited cell number by up to 30% (result not shown). For these reasons, all subsequent experiments were conducted using a 48-h treatment time.

The dose-dependence of the response to Dex was examined in rat calvaria primary cultures (Fig. 2A) and MC3T3-E1 cells (Fig. 2B). In both cell types, stimulation was observed over a physiological range of Dex, with primary cultures appearing to be somewhat more sensitive to hormone. Because the responsiveness of primary cultures and MC3T3-E1 cells to Dex was simi-

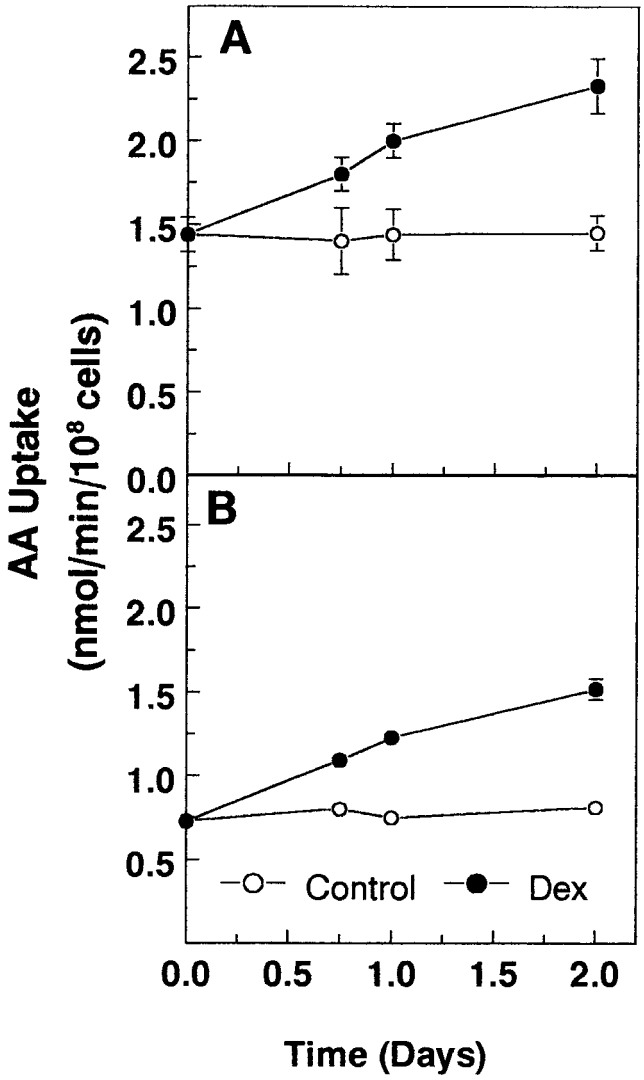


Fig. 1. Time course for dexamethasone (Dex) stimulation of ascorbic acid (AA) transport in primary rat calvarial osteoblasts (**A**) and MC3T3-E1 cells (**B**). Cells were isolated and grown as, described in Materials and Methods, in the presence or absence of 25 nM Dex. At the times indicated, [^{14}C]-L-AA uptake and cell number were measured.

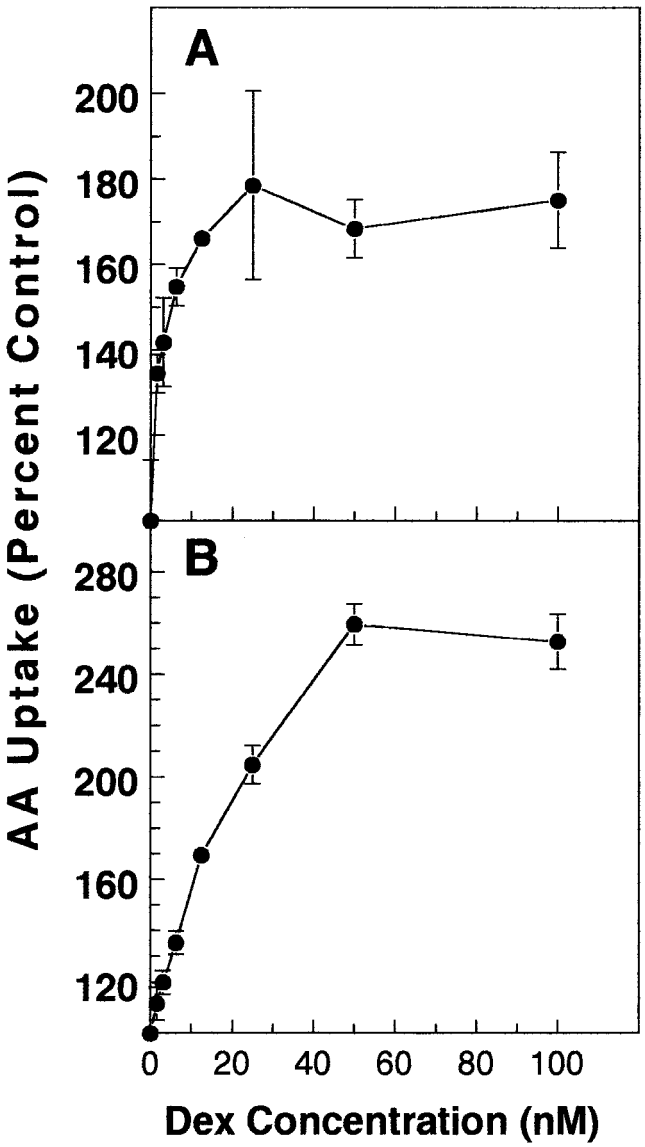


Fig. 2. Concentration dependence for Dex stimulation of AA transport in primary rat calvarial osteoblasts (**A**) and MC3T3-E1 cells (**B**). Cells were cultured for 2 days in the presence of the indicated concentration of Dex before being assayed for AA transport activity and protein. For each cell type, values are expressed as a percentage of the untreated control transport activity (primary calvarial osteoblasts, 78.2 ± 2.3 nmoles/min/g protein; MC3T3-E1 cells, 39.5 ± 1.1 nmoles/min/g protein).

lar, MC3T3-E1 cells were used to conduct a more detailed characterization of the hormone response.

The steroid specificity of the transport response was examined by treating MC3T3-E1 cells with 0.1 nM to 1 μM Dex, corticosterone, progesterone, and 17-β-estradiol (Fig. 3). Both Dex and corticosterone showed significant activity, with Dex being approximately 30 times more active (Dex EC_{50} = 10 nM; corticosterone EC_{50} = 300 nM). In contrast, both 17-β-estradiol and progesterone were inactive, even when they were administer at a concentration of 100 nM. The slight stimulation seen with 1 μM progesterone is consistent with reports indicating that it has weak glucocorticoid activity in certain systems (Rousseau et al., 1972).

Kinetics of AA transport

Kinetic constants for AA transport activity were determined for MC3T3-E1 cells grown in the presence or absence of 25 nM Dex (Fig 4). Uptake was measured in the presence of increasing concentrations of *L*-AA up to 100 μM, and data were fit to idealized Michaelis-Menten kinetics. To assess uptake of AA via a non-Na^+-dependent route, uptake was also measured in a separate set of cells by using transport buffer in which Na^+ was replaced with N-methylglucamine. At all AA concentrations, uptake under Na^+-free conditions was less than 2% of uptake in the presence of Na^+ and is not shown. The maximal velocity (V_{max}) of *L*-AA transport was 5.51 ± 0.04 nmol/min/10^8 cells for the control group, whereas Dex-treated cells exhibited a 2.5-fold

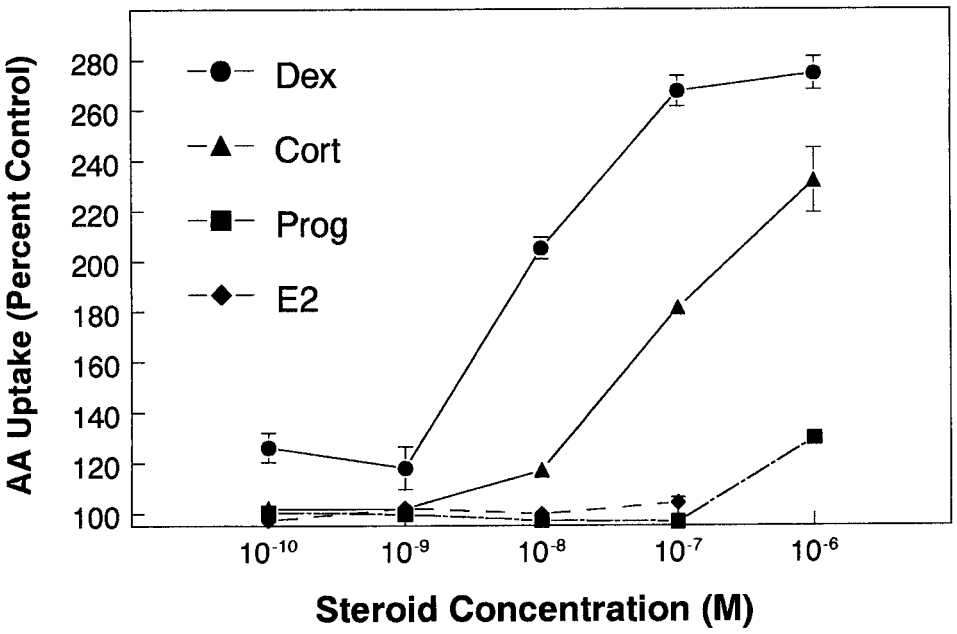


Fig. 3. Steroid specificity for glucocorticoid induction of AA transport in MC3T3-E1 cells. Cells were treated as described in Figure 2 with increasing concentrations of the indicated steroid hormone. Dex, dexamethasone; Cort, corticosterone; Prog, progesterone; E2, 17-β-estradiol. Control AA uptake, 0.67 ± 0.01 nmoles/min/10^8 cells.

greater V_{max} of 13.3 ± 0.29 nmol/min/10^8 cells. In contrast, the apparent K_m for control cells, 16.83 ± 0.39 μM, was not significantly different from the apparent K_m for Dex-treated cells, 17.74 ± 1.07 μM, and is in agreement with our previous report (Franceschi et al., 1995).

Requirement for protein synthesis

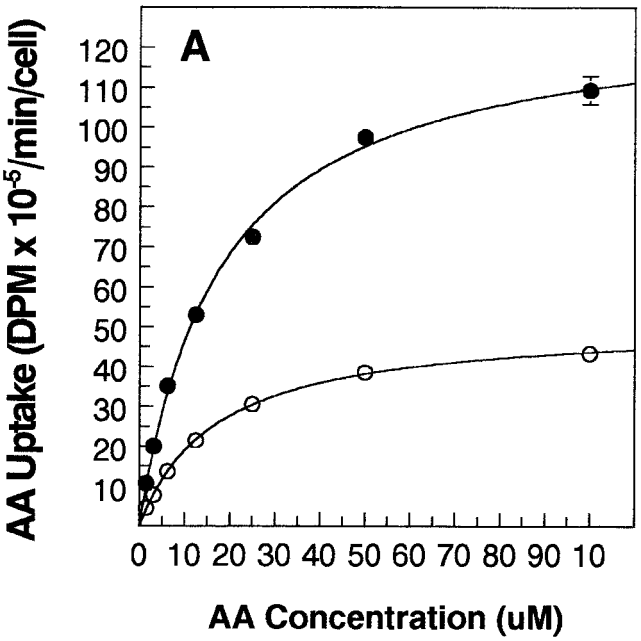
To determine whether protein synthesis is required for glucocorticoid stimulation of AA transport, cycloheximide was administered at 2.5 μg/ml to MC3T3-E1 cells grown for 24 h in the presence or absence of 25 nM Dex (Fig. 5). Control studies (not shown) demonstrated that this inhibitor concentration blocks greater than 95% of total protein synthesis in MC3T3-E1 cells. Cycloheximide did not affect AA transport in the control group but blocked the stimulatory activity of Dex. Results could not be explained by nonspecific toxic effects of the inhibitor on cells, because cycloheximide did not significantly reduce the number of cells per culture, yet it totally blocked the Dex-dependent increase in AA transport (cell numbers: Dex, 5.7 ± 0.4 × 10^5 cells/plate; Dex + cycloheximide, 5.1 ± 0.3 × 10^5 cells/plate).

Differential effects of Dex on AA and facilitative hexose transport

The Na^+-independent facilitative hexose transporter, GLUT-1, has been reported to provide a major route for AA uptake under certain conditions by serving as a transporter for dehydroascorbic (Vera et al., 1993, 1995). To assess the degree to which Dex selectively stimulated Na^+-dependent AA transport relative to this alternative pathway, MC3T3-E1 cells were grown in the presence or absence of 25 nM Dex and assayed for facilitative hexose transport, as measured by accumulation of the glucose analogue, 2-deoxy-*D*-glucose. Assays were conducted in transport buffer with and without Na^+. Figure 6 shows that 2-deoxyglucose transport was linear from 2.5 min to 30 min of incubation and, as expected, was not significantly affected by the presence or absence of Na^+ in the transport buffer. Dex-treated cells actually exhibited an approximate 50–60% reduction in 2-deoxyglucose transport activity compared with control cells, and this transport was Na^+-independent regardless of the treatment group. Thus, effects of Dex on AA vs. hexose transport are clearly distinguishable.

DISCUSSION

Glucocorticoids are potent stimulators of in vitro osteoblastic differentiation in primary cultures of calvarial osteoblast and marrow stromal osteoprogenitors (Bellows et al., 1987, 1990). They have been proposed to act by stimulating the expansion and differentiation of osteoprogenitors present in these mixed cell populations (Herbertson and Aubin, 1995; Ishida et al., 1996). The glucocorticoid-dependent up-regulation of AA transport we observed in primary cultures of rat calvarial osteoblasts represents an appropriate response to allow maximal induction of osteoblast differentiation, because, as we showed previously, AA-dependent collagen matrix synthesis is necessary for subsequent expression of osteoblast-related genes (Franceschi and Iyer, 1992; Franceschi et al., 1994), and osteoblast precursors must have an active Na^+-dependent AA transporter to respond to vitamin C (Franceschi et al., 1995). The association of high AA transporter activity with



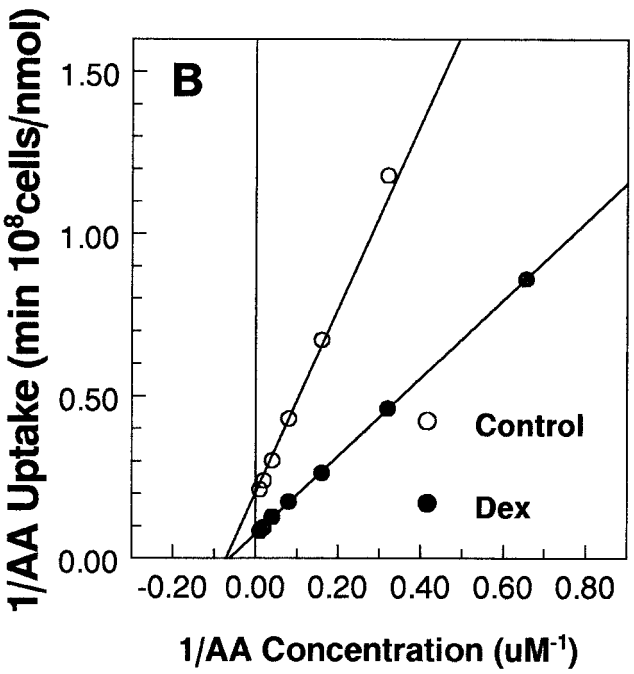


Fig. 4. **A,B:** Analysis of kinetic constants for control and Dex-treated cells. Cells were grown for 2 days in the presence or absence of 25 nM Dex. AA uptake was measured in the presence of the indicated concentrations of extracellular AA. Data were fit to idealized Michaelis-Menten kinetics. Derived kinetic constants were: control K_m = 16.83 ± 0.39 μM; Dex K_m = 17.74 ± 1.07 μM; control V_{max} = 5.51 ± 0.04 nmol/min/10^8 cells; Dex V_{max} = 13.1 ± 0.29 nmol/min/10^8 cells.

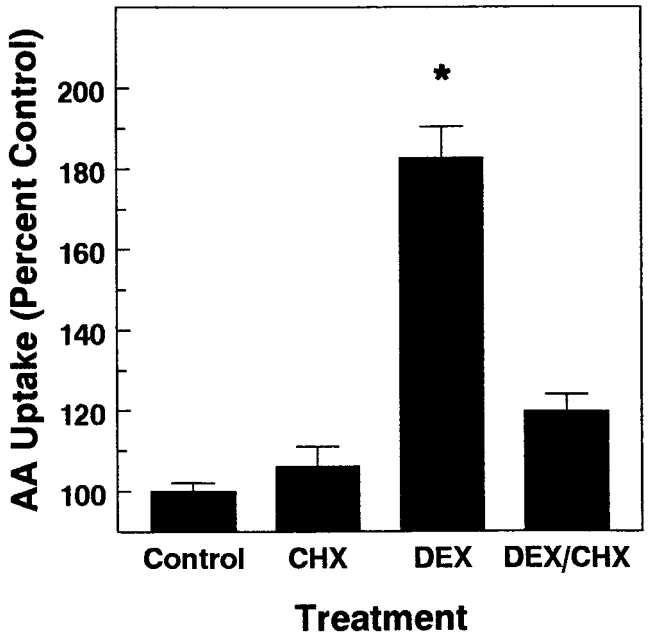


Fig. 5. Effect of cycloheximide (CHX) on induction of AA transport by Dex. Cells were cultured for 24 h with the indicated additions before measurement of AA transport activity. Control, no additions; DEX, 25 mM dexamethasone; CHX, 2.5 μg/ml cycloheximide. Control AA uptake, 0.75 ± 0.03 nmoles/min/10^8 cells.

osteoprogenitors/osteoblasts is consistent with the main function of this cell type, i.e., to synthesize an abundant collagenous ECM. Consistent with this concept, TGF-β, a growth factor known to increase ECM synthesis, can also stimulate AA transporter activity in UMR-106 rat osteosarcoma cells (Dixon and Wilson, 1992b).

We found that glucocorticoids also selectively stimulated AA transport in murine MC3T3-E1 cells. Although this immortalized cell line was useful for conducting a more detailed analysis of the glucocorticoid response, it was not possible to correlate changes in transport activity with increased osteoblast differentiation, which can be done with rat calvarial cultures. In fact, we observed that Dex treatment inhibited AA-dependent induction of osteoblast marker proteins and mineralization in MC3T3-E1 cells (Pandipati and Franceschi, unpublished). A recent report by Lian and coworkers (Lian et al., 1997) extensively analyzed the inhibitory activity of glucocorticoids on MC3T3-E1 cell differentiation and suggested that it may reflect either differences in the maturational state of these cells relative to primary cultures or a species-specific response of mouse osteoblasts to glucocorticoids. However, it is unclear why MC3T3-E1 cells share one response with primary rat calvarial osteoblasts (i.e., glucocorticoid stimulation of AA transport) yet exhibit an opposite response when differentiation is examined. One possible explanation is that the cell line, although it was originally clonal, has become heterogeneous as a result of passaging. The resulting mixed cell population is envisioned as being composed of both immature cells that exhibit an anabolic response to glucocorticoids (i.e., increased osteoblastic differentiation and AA transport) and other, more mature cells with a refractory transport response and differentiation that is inhibited by the steroid. We recently showed that the parent MC3T3-E1 cell line, in fact, is quite heterogeneous: Subclones were isolated with varying abilities to

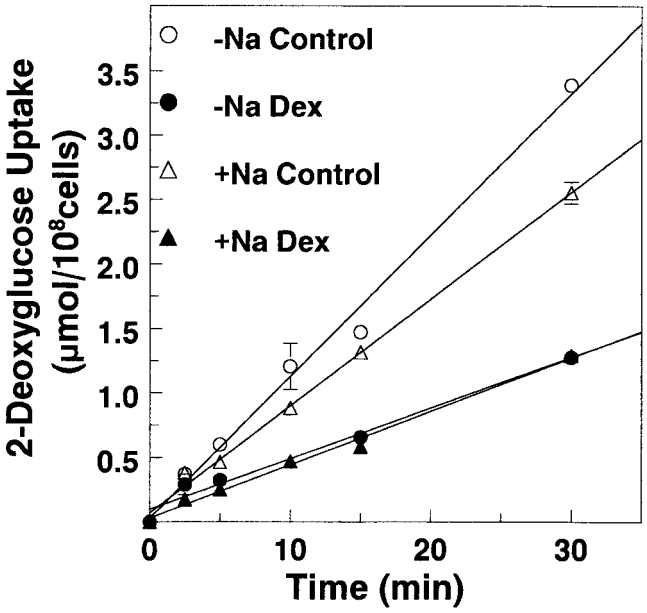


Fig. 6. Effect of Dex on 2-deoxyglucose transport activity. Cells were grown for 2 days in the presence or absence of 25 nM Dex. Uptake of 2-deoxy-[^{3}H]*D*-glucose was then measured after the indicated times by using transport buffer with or without Na^+.

differentiate in culture (Xiao et al., 1997). In addition, it is well established that the hormonal responsiveness of cells in the osteoblast lineage can vary with maturational state (Gerstenfeld et al., 1996). An alternative explanation for our results is that MC3T3-E1 cells, because they are an immortalized cell line, share properties with both mature and immature osteoblasts/osteoblast precursors.

Regardless of the explanation for these discrepancies, MC3T3-E1 cells clearly showed an AA-transport response after glucocorticoid treatment that was similar to primary rat calvarial cultures. The induction by glucocorticoids was rapid, occurring in less than 24 h; it was not related to changes in cell density; and it was specific, in that it was induced only by Dex and corticosterone, whereas related steroids (17-β-estradiol, progesterone) had minimal activity. The selective activity of Dex suggests that this response is mediated by the glucocorticoid receptor, which is present in MC3T3-E1 cells (Masuyama et al., 1992). Kinetic analysis revealed that Dex increased the V_{max} for AA transport by 2.5-fold without affecting K_m. When taken together with the results of studies using protein synthesis inhibitors, these experiments suggest that glucocorticoids act primarily by stimulating transporter expression. However, further investigations into the mechanism of this regulation will require cloning of the osteoblast Na^+-dependent AA transporter.

It has been suggested that dehydroascorbic acid is transported into cells primarily by the GLUT-1 transporter in a Na^+-independent fashion, after which, the dehydroascorbic acid is reduced to AA and accumulated at some intracellular site in a Na^+-dependent manner (Vera et al., 1993, 1995). Assuming that MC3T3-E1 cells follow a similar paradigm for AA transport, the stimulatory effects of Dex on AA transport should be explained by an up-regulation of the hexose transporter. However, 2-deoxyglucose transport was inhibited rather than stimulated by Dex treatment. This inhibition was seen regardless of whether 2-deoxyglucose accumulation was measured after short times (2.5 min), when initial hexose uptake is likely being measured, or after longer times (up to 30 min), when the accumulation of 2-deoxyglucose-6-phosphate predominates. The differential effect of Dex on AA vs. hexose transport, therefore, provides a means of resolving these two independent processes in bone cells. Previous studies by our laboratory (Franceschi et al., 1995) also found no competition between AA transport and 10 mM glucose or 10 mM 2-deoxyglucose and showed that cytochalasin B, an inhibitor of glucose transport, did not significantly reduce AA transport. In conclusion, although GLUT-1 may provide a route for vitamin C entry into certain cells, particularly those able to generate an oxidative environment and extracellular dehydroascorbate, it is unlikely that this transporter provides a major route for the accumulation of AA by osteoblasts.

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