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CHLOROQUINE ACCUMULATION AND ALTERATIONS OF PROTEOLYSIS AND PINOCYTOSIS IN THE RAT CONCEPTUS *IN VITRO*

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Abstract—The teratogenicity of chloroquine (CQ) has been hypothesized to result from its effects on lysosomal function, specifically the ability of the visceral yolk sac (VYS) to capture and degrade external macromolecules. Using the rat whole embroyo culture system, we evaluated the ability of CQ to accumulate in conceptal tissues and its effects on aspects of VYS function known to be important in the uptake and processing of nutrients. When CQ was added directly to the culture medium, it was found to accumulate rapidly in conceptal tissues, particularly the VYS. Tissue concentrations of CQ in the embryo proper reached approximately 10-fold those in the medium, whereas concentrations in the VYS exceeded by 100-fold the medium concentration within a 4-hr exposure on gestational day (GD) 10. Embryotoxic concentrations of CQ (10–30 μM) enhanced the activity of lysosomal cysteine proteinases measured *in vitro* under optimum pH conditions in both embryonic and VYS homogenates after a 26-hr treatment from GD 10–11. A different pattern of response in enzyme activity was observed between embryos and VYSs that could be attributed to the preferential accumulation of CQ in the VYS. Nonembryotoxic concentrations of CQ (1–7.5 μM) induced a concentration-dependent increase in VYS enzyme activity that peaked in conceptuses exposed to 20 μM CQ (an intermediate embryotoxic concentration). The enhanced cysteine proteinase activity was time dependent and appeared to increase gradually in conceptuses exposed to 10–20 μM CQ during the 26-hr culture period. This was in contrast to the rapid accumulation of CQ in conceptal tissues seen on gestational day 10. Protein content in the VYS was increased significantly after a 9-hr exposure of whole conceptuses to CQ (20 μM), indicating an inhibition of VYS proteolytic activity *in situ*. After 24 hr of exposure to 20 μM CQ, VYS protein content was not significantly different from control, but embryonic protein was reduced significantly by 20%. These observations are consistent with a model of reversible inhibition of VYS proteolysis by CQ followed by a compensatory increase in lysosomal proteinase activity. VYS fluid-phase pinocytosis was also assessed after CQ exposure and found to be inhibited only in the highest CQ concentration tested (30 μM). Lower concentrations of CQ that were still embryotoxic (10–20 μM) did not affect VYS fluid-phase pinocytosis, suggesting that inhibition of this activity is not primarily responsible for CQ embryotoxicity.

Key words: chloroquine; cysteine proteinases; histiotrophic nutrition; embryotoxicity

Chloroquine (CQ)†, an antimalarial drug, has been shown to be an *in vivo* teratogen in rodent and avian species [1–3], and at least one case report associates high dosages of CQ with human embryotoxicity [4]. Our recent work demonstrates that relatively low concentrations of CQ (10–30 μM) are embryotoxic to organogenesis-stage rat conceptuses grown *in vitro*, suggesting that direct effects of the drug on the conceptus could be responsible for the *in vivo* embyrotoxicity, rather than changes in the maternal system [5]. The actual cellular or molecular mechanisms by which CQ might produce embryotoxicity have not been investigated, although several authors have proposed that the ability of CQ to inhibit DNA repair accounts for the deleterious effects resulting from interaction between CQ and other teratogens [3, 6].

In contrast to the limited data available regarding the mechanisms of CQ embryotoxicity, substantial literature exists concerning the effects of the drug on mammalian cells *in vitro*. A large proportion of this work has focused on the function of the cellular vacuolar system because CQ is known to affect endocytosis, exocytosis and lysosomal function. CQ also has the ability to inhibit the processes of DNA synthesis and repair in cell-free systems [7]. The main effect of low concentrations of CQ (and related alkylamines) on mammalian cells, however, is generally agreed to be an increased pH of the normally acidic intracellular vacuoles (endosomes and lysosomes) resulting in changes in vesicular transport and enzymatic activity [for review, see Refs. 8 and 9]. Ohkuma and Poole [10] have

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† Abbreviations: CQ, chloroquine; VYS, visceral yolk sac; GD, gestational day; Z-Phe-Arg-7AMC, benzyloxycarbonyl - phenylalanine - arginine - 7 - amino - 4 - methyl-coumarin; Z-Phe-Ala-CHN_2, benzyloxycarbonyl-phenylalanine-alanine-diazomethylketone; PRIM, primaquine; FITC-dextran, fluorescein isothiocyanate-conjugated dextran; E-64, *trans*-epoxysuccinyl-leucylamido(4-guanidino)-butane; and MA, methylamine.

demonstrated that adding CQ to the medium of cultured macrophages rapidly and reversibly increases lysosomal pH. Increased vacuolar pH is believed to result from the trapping of CQ in these acidic organelles. The nonionized form of the compound readily diffuses across cell membranes at physiological pH, becoming ionized to a membrane-impermeant form within acidic compartments. Thus, protons are removed as the drug accumulates in acidic vacuoles, leading to an increased pH as vacuolar buffering capacity is exceeded. Vacuolar swelling occurs due to the osmotic effect of high concentrations of the weak base in these compartments, and possibly also from the accumulation of substrates such as proteins whose digestion by acid hydrolases is inhibited by CQ.

A common finding in studies of CQ-treated cells is an intracellular accumulation of protein resulting from decreased proteolytic degradation of endocytosed exogenous proteins [11–13]. This effect has typically been attributed to inhibition of lysosomal proteolysis by CQ, due to either increased lysosomal pH or possibly the direct inhibitory effects of the drug on proteinases [14]. Indeed, susceptibility of cellular hydrolytic capacity to inhibition by CQ and related compounds has usually been taken as evidence of lysosomal involvement. There is also evidence, however, to support the idea that CQ can inhibit vacuolar trafficking [15] and block receptor recycling [16]. The cellular effects of CQ, therefore, include alterations in the normal intracellular uptake, trafficking, degradation and secretion of several macromolecules. These changes in the function of the cellular vacuolar system in various cell types *in vitro*, together with the reported ability of CQ to inhibit endocytosis and lysosomal proteolysis in the cultured GD 17 rat VYS [17], suggest that the mechanism of CQ embryotoxocity might involve the inhibition of VYS-mediated nutritional function during organogenesis. This function depends upon the capacity of the VYS endoderm epithelium to endocytose and degrade exogenous proteins and is essential for embryonic growth during this sensitive period of development [18, 19]. The evidence indicates that VYS-mediated nutrition is a target for several embryotoxicants that affect this pathway [for review, see Ref. 20].

Previous work in this laboratory has implicated the VYS endoderm epithelium as a main target for CQ, although we suspect that the embryo proper might also be directly affected [5]. At medium concentrations of 30 μM, CQ increased VYS protein content even though the DNA content of the same tissue decreased. This treatment also produced an accumulation of eosinophilic material (presumably protein) in cytoplasmic vacuoles of the VYS epithelium, the normal site of degradation of exogenous protein endocytosed by these cells [21]. Such findings are characteristic of lysosomal dysfunction and suggest that CQ may inhibit lysosomal proteolysis in the VYS endoderm, the process which the embryo relies upon for its amino acid supply. The lysosomal cysteine proteinases cathepsin B and cathepsin L have been implicated as being responsible for VYS proteolysis, mainly because of the ability of leupeptin to inhibit this process [22, 23].

The objective of the present study was to investigate the ability of CQ to accumulate in conceptal tissues and inhibit several aspects of VYS nutritional function believed to be important for embryonic nutrient supply during organogenesis. These parameters include the activities of conceptal lysosomal cysteine proteinases (cathepsin B and cathepsin L) and fluid phase endocytosis. This report provides data to support the idea that CQ embryotoxicity results from accumulation of the drug in conceptal tissues with subsequent alterations of VYS (and possibly embryonic) vacuolar systems.

MATERIALS AND METHODS

Chemicals. The cysteine proteinase substrate Z-Phe-Arg-7AMC, 7AMC standard and the cysteine proteinase inhibitor were obtained from Bachem Bioscience Inc., Philadelphia, PA. Chloroquine diphosphate, PRIM, FITC-dextran, MA and E-64 were obtained from the Sigma Chemical Co., St Louis, MO. All other reagents were of the highest quality commercially available.

Animals. Time-mated primigravida Sprague–Dawley rats were obtained from the Reproductive Sciences Program Small Animal Core Facility, the University of Michigan, on GD 7–9. The morning following copulation, as indicated by a sperm positive vaginal smear, was designated as GD 0. Pregnant animals were maintained on a 14-hr light/10-hr dark cycle and given free access to Purina Rat Chow (Ralston Purina) and water until the morning of explantation.

Embryo culture. On the morning of GD 10, pregnant dams were anesthetized with ether and exsanguinated via the abdominal aorta. The blood was centrifuged and the serum heat-inactivated and stored at −20° for subsequent use in culture medium. Uteri were removed from the dams and placed in warmed Hanks' Balanced Salt Solution (HBSS, Gibco BRL, Gaithersburg, MD), and the implantation sites were dissected free. Conceptuses were removed carefully from the decidual mass, and Reichert's membrane was opened to allow the embryo to develop properly in culture. Conceptuses were placed in 125-mL roller bottles containing 33% heat-inactivated female rat serum in HBSS supplemented with penicillin G (100 IU/mL) and streptomycin (50 μg/mL). The medium had been warmed to 37° and gassed with 20% O_2/5% CO_2/75% N_2 prior to addition of conceptuses. Less than ten conceptuses/bottle were cultured in a total volume of 15 mL culture medium. The bottles were sealed and placed in a roller incubator overnight and then were regassed with 95% O_2/5% CO_2 on the morning of GD 11. For DNA, protein, and enzyme assays, whole conceptuses were removed from the culture medium at various times and rinsed three times in HBSS. The embryos and VYSs were dissected apart and placed individually in enough sodium phosphate buffer (50 mM, 1 mM EDTA, pH 6.0) to produce a homogenate of 200–300 μg protein/mL. The tissues were disrupted ultrasonically and frozen at −70° prior to the protein assays.

Cathepsin B and L activity was determined after the same tissue was taken through a second freeze–thaw and briefly centrifuged to remove insoluble material.

Exposure. Stock CQ solutions of 15 mg/mL were prepared fresh in sterile HBSS, pH 7.4. The stock or vehicle (total volume of 15 μL or less) was added directly to the culture medium immediately prior to the addition of conceptuses on the morning of GD 10, and exposure was continuous throughout the culture period.

Protein and DNA assays. Protein content was determined by the method of Bradford [24], using bovine gamma globulin standard and dye reagent supplied by Bio-Rad (Richmond, CA). The assay was modified for microtiter plate assay as described by Stark *et al.* [25]. DNA content was determined spectrofluorometrically by the method of Labarca and Paigen [26], using bovine DNA as a standard.

Chloroquine extraction and determination. To estimate CQ concentrations present in conceptal tissues exposed to the drug in whole embryo culture, an adaptation of the method of Adelusi and Salako [27] was employed. This assay utilizes an ether extraction of CQ from a basic solution with back extraction into acid after two wash steps. The fluorescence of the extracted CQ is then read in a buffered medium at pH 9.5 with excitation and emission set to 331 and 386 nm, respectively. The second wash step, designed to remove metabolites, was omitted from our assay since it was found to have no effect upon our determination of CQ content in tissues as calculated from authentic CQ standards extracted concurrently. The assay was sensitive enough to detect low micromolar concentrations of CQ, so that determinations could be made on individual embryos and VYSs. Untreated control tissues consistently gave readings equivalent to the zero standard while recovery of CQ from spiked tissue samples approached 90%. To calculate original tissue concentrations, a factor was determined in order to take into account the volume of the tissue extracted. Measurements of tissue wet weight were therefore performed on control tissues (tissues were dissected in HBSS, held gently between watchmakers forceps, and any excess water was blotted; the blotted tissues were placed into preweighed microcentrifuge tubes and then reweighed, from which the tissue wet weight was determined). It was assumed that (1) the tissue density was 1.0 g/mL; and (2) the volume of the treated tissues was equal to that of the controls.

Cathepsin B and L activity. The combined hydrolytic activity of the lysosomal cysteine proteinases cathepsin B and cathepsin L was determined using the fluorometric substrate Z-Phe-Arg-7AMC. Both enzymes can hydrolyze this substrate effectively [28], and both have been implicated as likely targets for proteinase inhibitors in the rat VYS [22, 29]. Aliquots of the tissue homogenates were diluted and used as the enzyme source. The assay was a slight modification of the method of Barrett and Kirschke [28] for cathepsin L, substituting 0.1% Triton X-100 for the Brij 35 diluent used in the original assay and determinations were made at 37° rather than 30°. All other conditions were as originally described. Since Z-Phe-Arg-7AMC can also be hydrolyzed by some trypsin-like enzymes, it was important to demonstrate that the enzyme activity present in our homogenates was due to lysosomal cysteine proteinases. Experiments showed that the activity present in our samples was latent (activated by freeze–thaw), enhanced at low pH and in the presence of sulfhydryl reducing agents (data not shown) as well as being strongly inhibited by specific inhibitors of cathepsins B and L such as E-64 and the peptidyl diazomethylketones (see Fig. 2 and Results). These properties are characteristic of lysosomal cysteine proteinases [28, 30].

Grubb *et al.* [31] have studied the ontogeny of lysosomal cysteine proteinase activity in the rat VYS during the second half of gestation and found two enzymes capable of hydrolyzing Z-Phe-Arg-7AMC, presumably cathepsins B and L. Their data show that GD 12.5 VYSs possess very low cathepsin B activity compared with cathepsin L activity. In the present study, we have not distinguished between these two closely related enzymes, but further characterization studies indicate that the activity measured in our assay is also mainly due to cathepsin L.*

Pinocytosis assay. Fluid-phase pinocytosis was assayed by exposing viable conceptuses in culture to an FITC-dextran polymer for various time periods after which the amount of FITC fluorescence accumulated by the VYS was measured spectrofluorometrically. FITC-dextrans are known to accumulate in the lysosomal compartment and have been used as tracers of fluid-phase pinocytosis in various systems [32, 33]. We have found that accumulation of FITC fluorescence by the VYSs of viable conceptuses exposed to FITC-dextrans is characteristic of fluid-phase marker uptake in the rat VYSs as defined by Duncan and Lloyd [34], being linear with respect to time, partially inhibited by colchicine, and completely inhibited by incubation at 4° (data not shown). Another characteristic of fluid-phase pinocytosis observed in conceptuses incubated with FITC-dextran is that only the VYS (not the embryo proper) accumulates FITC fluorescence. In addition, fluorescent microscopy of VYSs exposed to FITC-dextran has confirmed that FITC-fluorescence is localized within discrete vacuoles in the cytoplasm of the VYS endoderm epithelium (not shown).

Conceptuses exposed to culture medium containing 10 μM FITC-dextran for various times were removed from culture, rinsed four times in ice-cold 0.9% NaCl and their VYSs dissected free. Individual VYSs were then placed in 0.5 mL HBSS and ultrasonically disrupted. An aliquot of the resulting homogenate was saved for protein determination. Another 250-μL aliquot was added to an equal volume of 0.1% Triton X-100 and mixed. This mixture was diluted with 2 mL of 50 mM sodium phosphate buffer, pH 7.4, and fluorescence was measured at FITC wavelengths (excitation 495 nm; emission 520 nm). Arbitrary fluorescence units were corrected for background fluorescence of VYSs not exposed to the dextran and normalized to the protein content of each respective VYS. By also assaying the fluorescence

* Ambroso JL and Harris C, unpublished results.

Table 1. Chloroquine accumulation in conceptal tissues following exposure in whole embryo culture

Treatment*	Chloroquine extracted† (μg)	Tissue chloroquine concentration‡ (mM)
4 hr		
Day 10 embryo	0.2 ± 0.0	0.3 ± 0.1 (6)
Day 10 yolk sac	1.7 ± 0.4	5.6 ± 1.2 (6)
Day 10 whole embryo	2.6 ± 0.6	1.4 ± 0.3 (3)
26 hr		
Day 11 embryo	1.0 ± 0.3	0.4 ± 0.1 (4)
Day 11 yolk sac	4.7 ± 0.7	4.6 ± 0.6 (7)
Day 11 whole embryo	7.4 ± 0.5	0.8 ± 0.1 (3)

* Initial concentrations of 30 μM chloroquine were added directly to the culture medium at the start of the culture period (GD 10). After a 4- or 26-hr incubation, conceptuses were removed from the culture medium, rinsed twice in HBSS, and either dissected to embryos and VYSs or left intact.

† Chloroquine was extracted from individual tissues, as described in Materials and Methods. Data represent means ± SD.

‡ Concentrations were calculated based on the volume of control tissues, as described in Materials and Methods. Data are means ± SD of (N) samples from three separate experiments.

present in the culture medium it is possible to calculate an Endocytic Index, which represents the amount of culture medium cleared of the tracer molecule per mg VYS protein per hour [35]. Using this procedure, an Endocytic Index of approximately 3.0 μL/mg/hr can be calculated for GD 11 VYSs exposed to the 40-kDa FITC-dextran. This value is within the range of published endocytic indices for rat VYS using radiolabeled substrates.

Statistical analysis. The statistical software package Statview (Abacus Concepts, Inc., 1986) was used to analyze enzyme activity data and Endocytic Index data. One-way analysis of variance was used to determine overall differences between multiple treatment groups. This was followed by the Scheffe test for multiple comparisons of differences between individual treatment means. Where only one treatment group was present (Figs. 3 and 5), the data were analyzed using an unpaired *t*-test to compare two means at particular time points.

RESULTS

The ability of rat conceptuses to accumulate CQ from the culture medium was assessed by acid extraction of CQ from conceptal tissues, as described in Materials and Methods. The data in Table 1 illustrate that both embryos and VYSs of conceptuses exposed to CQ attain concentrations of CQ much greater than those present in the culture medium. This is particularly true of the VYS. Tissue concentrations estimated after a 26-hr exposure to 30 μM CQ (0.4 mM in the embryo; 4.6 mM in the VYS) were not substantially different from those determined after 4 hr of exposure (0.3 mM in the embryo and 5.6 mM in the VYS). It should be noted that the use of control tissue wet weights to calculate CQ concentrations in treated tissues may overestimate the CQ concentration because CQ causes cytoplasmic swelling and likely increases tissue volume. Some of this effect, however, may be compensated for by decreased overall growth observed in CQ-treated conceptuses.

As observed previously [5], CQ exposure throughout a 26-hr embryo culture period elicited a differential response between embryos and VYSs in terms of protein content (Fig. 1A). Embryonic protein content showed a concentration-dependent decrease (to 57% of control at 30 μM, $P < 0.001$), whereas VYS protein content showed an inverse concentration–response relationship, being decreased only in the 10 μM treatment group (85% of control, $P < 0.05$). VYS protein content was decreased slightly by 20 μM CQ treatment (91% of control, not significant) but was increased significantly in conceptuses in the 30 μM CQ group (126% of control, $P < 0.001$). This contrasts sharply with the observed concentration-dependent decrease in DNA content of both embryos and VYSs in the same conceptuses (Fig. 1B).

The combined activities of cathepsins B and L in GD 11.5 conceptuses cultured in the presence of CQ are also presented in Fig. 1. Panels C and D show this activity normalized to either protein or DNA content, respectively. A comparison of these panels indicates that the traditional normalization of enzyme activity to protein content underestimated VYS cathepsin activity in the 20 and 30 μM treatment groups because of the abnormal accumulation of protein in VYSs at these higher concentrations. In the 10 μM group, however, normalization of enzyme activity to either protein or DNA content resulted in little change in activity with reference to control (333 vs 337% of control, respectively). Subsequent enzyme activities, therefore, are presented normalized to DNA content for tissues exposed to CQ concentrations greater than 10 μM.

As expected, cathepsin B and L activities in the

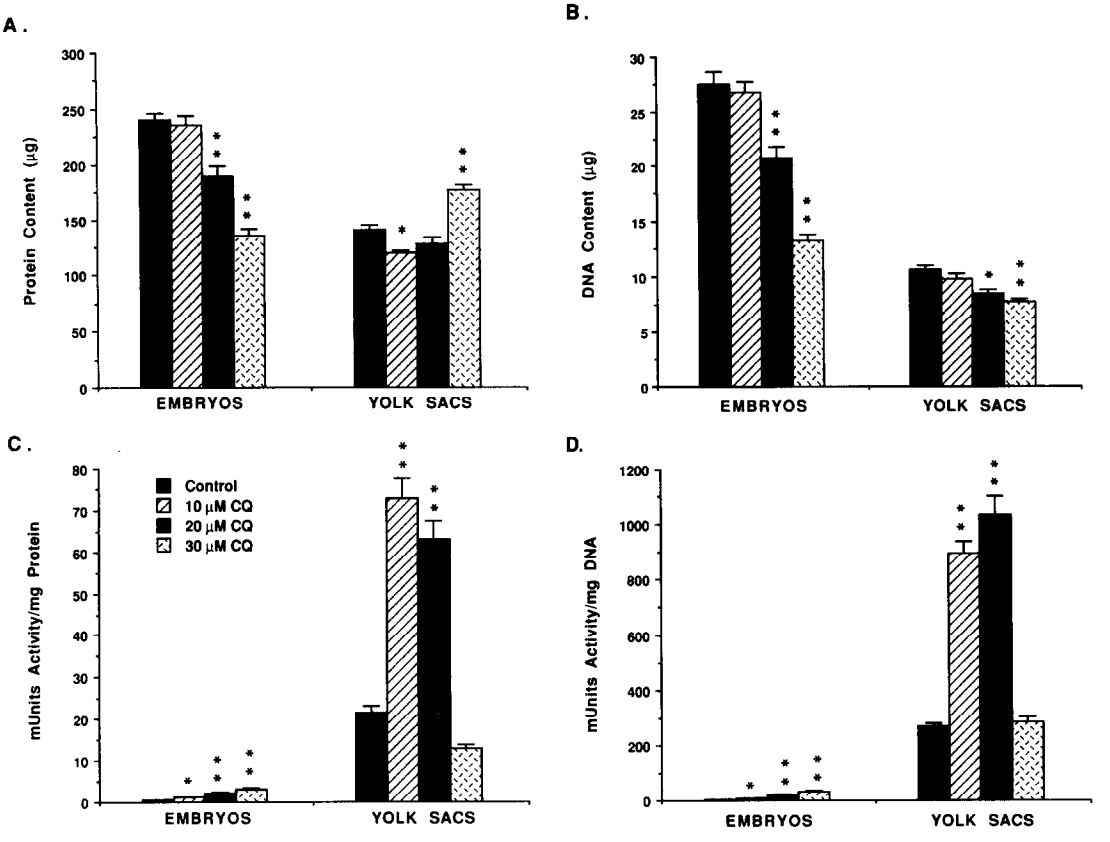

Fig. 1. Protein content (A), DNA content (B) and cathepsin B and L activity normalized to protein (C) and DNA (D) content of conceptal tissues at the end of a 26-hr culture period (GD 11.5). Mean embryonic enzyme activity was 0.5, 1.2, 2.2 and 3.2 mU/mg protein and 4, 10, 20, and 32 mU/mg DNA for control, 10 μM, 20 μM, and 30 μM CQ-treated groups, respectively. Bars represent means ± SEM of 17–23 samples from three replicate experiments. The pattern legend in panel C applies throughout the figure. Key: (*) $P < 0.05$, and (**) $P < 0.001$ (Scheffe multiple comparison test).

VYS were much greater overall than those observed in the embryo proper (mean = 269 mU/mg DNA for control VYSs vs 4 mU/mg DNA for control embryos) when measured at the end of the culture period. Twenty-six-hour exposure of conceptuses to embryotoxic concentrations of CQ (10–30 μM) enhanced the lysosomal cysteine proteinase activities in both embryos and VYSs of GD 11.5 conceptuses. The enhancement was greatest in embryonic tissues, where 30 μM CQ treatment increased activities to 803% of control (32 mU/mg, $P < 0.001$). Although the absolute levels of enzyme activity in the VYS were much greater than those in the embryo proper, the CQ-induced enhancement of activity in the VYS was not as great: maximum activities in VYSs exposed to 20 μM CQ reached 385% of control when normalized to DNA content (1035 mU/mg, $P < 0.001$). Another difference in the CQ-induced enhancement of cathepsin activity observed between embryos and VYSs was in the concentration–response relationship. While CQ treatment caused a concentration-dependent increase in enzyme activity in the embryo proper that peaked in conceptuses exposed to 30 μM CQ, the peak activity in the VYS occurred in conceptuses exposed to 20 μM CQ and decreased to 106% of control in the 30 μM group (286 mU/mg, not statistically significant).

Further experiments were performed to characterize the CQ-induced enhancement of cathepsin B and L activity in conceptal tissues. These experiments included evaluation of various exposure times and concentrations of CQ and related lysosomotropic compounds, as well as the use of specific inhibitors to verify lysosomal cysteine proteinase activity. Figure 2 shows a 30% decrease in the specific activity of cathepsin B and L between GD 10.5 and GD 11.5 in control VYSs of conceptuses grown *in vitro*. This is a consistent finding that has also been observed in freshly explanted tissue (data not shown). In VYSs of conceptuses exposed to minimally embryotoxic concentrations of CQ or PRIM for 2 hr on GD 10, no significant change in enzyme activity occurred compared with concurrent controls, whereas 24-hr exposure elicited significantly enhanced activity (240% of control, $P < 0.001$ and 170% of control, $P < 0.05$ for CQ and PRIM, respectively). This enhanced activity produced by

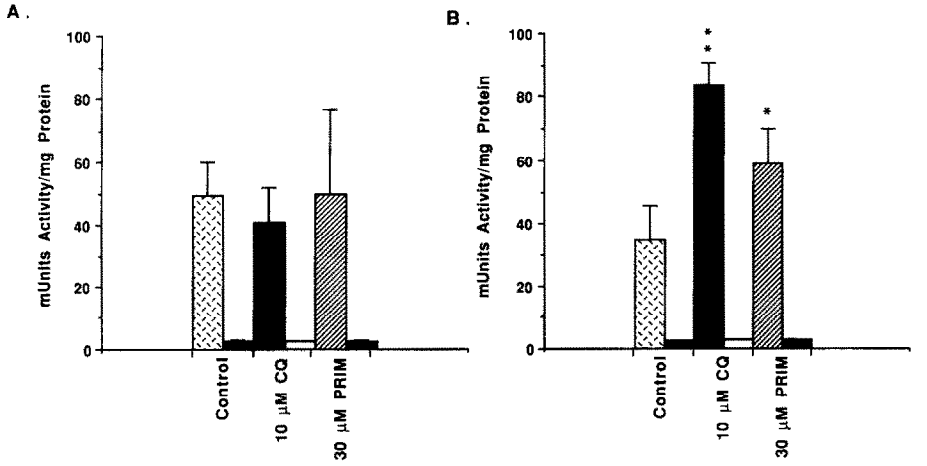

Fig. 2. Characterization of enhanced VYS cathepsin B and L activity in conceptuses exposed to lysosomotropic weak bases. Bars represent means ± SD of N = 4 samples from one experiment. (A) Activity in VYS homogenates from GD 10.5 conceptuses exposed to minimally embryotoxic concentrations of either chloroquine (CQ) or primaquine (PRIM) for 2 hr. Cysteine proteinase activity is confirmed in the right-hand column of each group by inhibition with 1 μM Z-Phe-Ala-CHN_2 (5-min pretreatment at 37°). (B) Activity in VYS homogenates from GD 11.5 conceptuses grown for 26 hr in the same medium as used in "A." As in "A," the right-hand column of each group was inhibited with 1 μM Z-Phe-Ala-CHN_2. Key: (*) $P < 0.05$, and (**) $P < 0.01$ (Scheffe multiple comparison test).

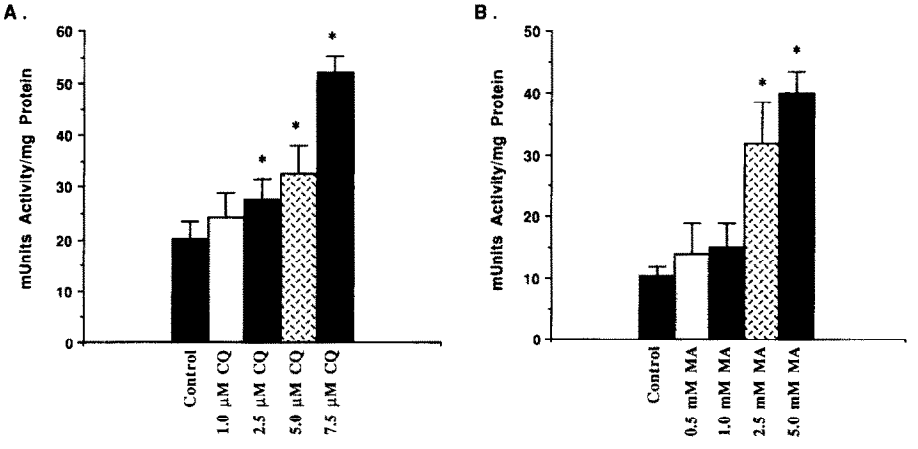

Fig. 3. Effect of low concentrations of lysosomotropic amines on VYS cathepsin B and L activity. Cultured rat conceptuses (GD 10.5 to 11.5) were exposed for 26 hr to concentrations of less than 10 μM CQ (A) or less than 6 mM MA (B). Bars represent means ± SD of 4–8 samples from two replicate experiments (A) and 3–6 samples from one experiment (B). Key: (*) $P < 0.05$ (Scheffe multiple comparison test).

CQ and PRIM treatment was found to be sensitive to inhibition by low micromolar concentrations of specific cysteine proteinase inhibitors such as Z-Phe-Ala-CHN_2 (Fig. 2). Compound E-64 was also extremely effective, inhibiting control and CQ-enhanced activities nearly 100% at 5 μM concentrations (data not shown). These data indicate that the CQ-induced enhancement of cathepsin activity was not artifactual. Figure 3 illustrates that a 26-hr exposure to non-embryotoxic concentrations of CQ (below 10 μM) enhanced cathepsin B and L activity in VYSs in a concentration-dependent fashion. This was also true of MA, another lysosomotropic amine (Fig. 3B). The concentrations of PRIM and MA used in Figs. 2 and 3 showed little toxicity and did not affect VYS protein content. These data are therefore shown normalized to protein content.

In Fig. 4 we see the effect of exposure time on cathepsin B and L activity and protein content of conceptuses treated with 20 μM CQ. The CQ-induced increases in enzyme activity were detectable

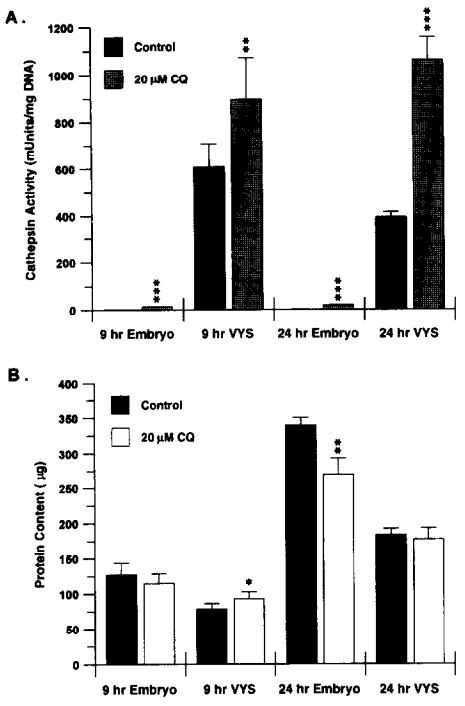

Fig. 4. Lysosomal cysteine proteinase activity (A) and protein content (B) of organogenesis-stage conceptuses exposed to 20 μM CQ for 9 or 24 hr. Mean embryonic enzyme activities were 2.7 (control) and 14.1 (20 μM CQ) mU/mg DNA after a 9-hr incubation on GD 10.5; and 2.0 (control) and 19.3 (20 μM CQ) mU/mg DNA after a 24-hr incubation on GD 11.5. Bars represent means ± SD of 4 samples from one experiment. Key: (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$ (*t*-test).

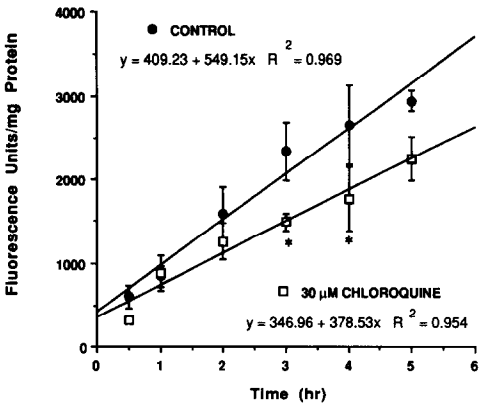

Fig. 5. Effect of chloroquine on fluid-phase pinocytosis in GD 10.5 VYSs. Points represent means ± SD of 3–6 samples from three experiments. Key: (*) $P < 0.05$ (*t*-test).

Table 2. Effect of chloroquine on VYS fluid-phase pinocytosis in rat conceptuses *in vitro**

	Endocytic Index (μL/mg/hr)
8-hr Treatment	
GD 10.5 control (8)	3.4 ± 0.5
10 μM CQ (7)	3.7 ± 0.6
20 μM CQ (8)	3.3 ± 0.7
30 μM CQ (8)	2.1 ± 0.9†
24-hr Treatment	
GD 11.5 control (15)	2.8 ± 0.3
10 μM CQ (15)	3.1 ± 0.4
20 μM CQ (15)	2.5 ± 0.3
30 μM CQ (6)	1.8 ± 0.2‡

* Chloroquine and 40 kDa FITC-dextran were added simultaneously to the culture medium and incubated for the time indicated. The Endocytic Index was calculated by the method of Williams *et al.* [35], and represents the mean ± SD of (N) yolk sacs taken at the time indicated.

†,‡ Significantly different from control; † $P < 0.01$ and ‡ $P < 0.001$ (Scheffe multiple comparison test).

after 9 hr of treatment in both embryos and VYSs, although the magnitude of the increase was much less than that observed after 24 hr (VYS activity was increased by 48% after 9 hr, $P < 0.01$, vs a 170% increase after 24 hr, $P < 0.001$). Interestingly, 20 μM CQ treatment elicited an increase in VYS protein content after 9 hr (118% of control, $P < 0.05$) that was not observed after 24 hr of treatment (96% of control, not significant).

The effect of CQ treatment on VYS function in cultured conceptuses was evaluated further by determinations of fluid-phase pinocytosis. Uptake of FITC-dextran was measured during several periods between GD 10 and GD 11 in embryo culture. Conceptuses exposed to FITC-dextran accumulated FITC fluorescence only in the VYS and not the embryo proper, which is characteristic of fluid-phase uptake in whole conceptuses (data not shown). Uptake of FITC-dextran by the VYSs was linear for at least 5 hr in GD 10.5 conceptuses (Fig. 5). Linearity was also observed under similar conditions in day 11.5 conceptuses (data not shown). One-hour pretreatment of GD 10.5 conceptuses with 30 μM CQ, followed by a 4-hr exposure to the FITC-dextran in the presence of 30 μM CQ, inhibited the accumulation of FITC fluorescence in VYSs by approximately 35% ($P < 0.05$). Table 2 lists Endocytic Indices calculated from the uptake of FITC-dextran by VYSs. The highly embryotoxic concentration of 30 μM CQ was again observed to inhibit VYS fluid-phase pinocytosis, as evidenced by the decreased Endocytic Index (60% of control after 8 hr, $P < 0.01$; 66% of control after 24 hr; $P < 0.001$). Lower concentrations of CQ caused small changes in the Endocytic Index, which were not statistically significant. Because these data (like enzyme activity) are normalized to protein content, these decreases in fluid-phase pinocytosis can be attributed largely to the increased VYS protein content produced by 30 μM CQ treatment. We are currently unable to normalize these data to DNA content because fluorescein interferes with our DNA determination method. The protein content in VYSs exposed to 30 μM CQ was consistently higher than in control

VYSs by 20–25%, and thus any true decreases in fluid-phase uptake by CQ are small (15% or less).

DISCUSSION

The theory of lysosomotropism, as proposed by de Duve *et al.* [8], states that weak bases such as CQ can accumulate in acidic cellular compartments by virtue of their physico-chemical properties. These authors determined that cellular CQ concentrations could rapidly reach 10 mM when rat fibroblasts were exposed to culture medium containing 100 μM CQ. It is presumed that the concentration of the drug in the lysosomes is even greater. The data presented here illustrate that a similar phenomenon occurred when rat conceptuses were exposed to CQ during whole embryo culture. Conceptuses were found to accumulate high concentrations of the drug within their tissues relatively early in the culture period, and concentrations determined at the end of the culture period were also high. These findings suggest that CQ concentrations in conceptal tissues reach an apparent steady state rapidly, as has been observed in cell culture experiments [14]. The VYS attains much higher concentrations of CQ than the embryo proper, which seems reasonable considering the extensive vacuolar system of the VYS endoderm epithelium and its direct exposure to the culture medium.

High concentrations of CQ can alter the lysosomal milieu and lead to a condition resembling "lysosomal storage disease," in which the lysosomes accumulate macromolecules which are otherwise normally degraded and removed [36]. We have found that exposure of rat conceptuses to highly embryotoxic concentrations of CQ (30 μM) increases the protein content of the VYS, and this increased protein most likely accumulates within lysosomes of the VYS endoderm [5]. These data suggest that CQ acts similar to other protease inhibitors such as leupeptin, resulting in accumulation of protein in the VYS epithelium [23]. Although the VYSs of conceptuses exposed to lower concentrations of CQ that are still embryotoxic (10 and 20 μM) did not exhibit increased protein content at the end of the culture period, increases in VYS protein content could be detected at intermediate time points. This observation would argue for a transient inhibition of VYS proteolysis by CQ that is sufficient to cause embryotoxicity.

In light of these observations, our current data may appear contradictory by showing an increase, rather than a decrease, in lysosomal proteinase activity (Figs. 1–4). Our hypothesis states that CQ inhibits conceptal proteolysis reversibly *in situ* via increased lysosomal pH or osmotic effects as described previously. Once the tissue is homogenized and diluted in assay buffer under conditions for determination of optimal activity, we would no longer expect CQ to inhibit the enzymes. Thus, the increases in cathepsin B and L activity observed *in vitro* following conceptal exposure to CQ likely represent a compensatory increase in enzyme activity. It seems reasonable that in conceptuses exposed to nonembryotoxic concentrations of CQ (less than 10 μM), the increased cathepsin B and L activity is sufficient to compensate for the inhibitory effects of CQ. However, once the medium concentration of CQ reaches 10 μM, increased VYS cysteine proteinase activity can no longer compensate fully for the altered lysosomal milieu, and embryotoxicity results. Because increases in enzyme activity occur relatively slowly compared with the accumulation of CQ in conceptal tissues, there also appears to be a period of time during which VYS proteolysis is likely to remain interrupted by CQ treatment.

A model of reversible inhibition and compensatory increase in proteolytic activity is supported by reports in the literature. The ability of CQ to inhibit degradation of both endogenous and exogenous proteins has been well established [12–17, 37] and its inhibition of proteolysis in perfused rat kidney is readily reversible [38]. Moreover, long-term administration of reversible protease inhibitors has been shown previously to enhance lysosomal protease activity when measured *in vitro* [39, 40]. At present, direct experimental evidence that CQ inhibits VYS proteolysis *in situ* in our system is lacking, but we are developing methods to assess this possibility.

Enhanced cathepsin B and L activity in conceptal homogenates indicates that treatment with CQ or related compounds either stimulates enzyme synthesis, inhibits enzyme turnover, or both. In this regard, Kominami *et al.* [41] have described increased *content* and half-life of cathepsin B and L in rat liver following *in vivo* administration of E-475 (an irreversible cysteine proteinase inhibitor), which was not blocked by cycloheximide. These authors suggest that lysosomal cysteine proteinases may be involved in their own degradation. However, increased proteolytic activity and autophagy have also been observed in several cell types as a response to amino acid withdrawal [reviewed in Ref. 42], indicating that regulatory synthetic mechanisms might also be available. Further experiments are required in order to distinguish between these possibilities in the rat conceptus.

Thirty micromolar CQ treatment maximally enhanced the cathepsin B and L activity measured in embryos, whereas that in VYSs was found to be no different than in the controls. This differential response can be attributed logically to the ability of the VYS to accumulate much higher concentrations of the drug than the embryo proper. The inability of 30 μM CQ treatment to enhance VYS cathepsin B and L activity over control also represents a qualitative change in the action of CQ at this concentration which could result from several potential effects of the drug: (1) irreversible inhibition of the enzyme if lysosomal pH rises above 7, since cathepsin L is inhibited irreversibly above neutral pH [43]; (2) direct inhibition of cathepsin B and L by high intralysosomal concentrations of CQ, as has been observed for cathepsin B *in vitro* [14]; (3) altered targeting of the enzymes to the lysosome (via the mannose-6-phosphate receptor), as occurs in CQ-treated fibroblasts [44]; and (4) inhibition of protein synthesis due to decreased intracellular amino acid pools as seen in several cell types [37, 45].

In addition to alterations in lysosomal hydrolytic capacity, the effects of CQ on mammalian cells include changes in endocytotic capacity. Reduced

VYS pinocytosis has been reported previously after exposure of cultured GD 17.5 VYSs to higher concentrations of CQ than those used in the present study [17]. Our data indicate that VYS fluid-phase pinocytosis is diminished in CQ-treated conceptuses at concentrations that are highly embryotoxic (30 μM), but much of this effect can be attributed to the normalization of these data to increased VYS protein content. It therefore appears that reduced fluid-phase pinocytosis in the VYS may contribute to the embryotoxicity of CQ only at concentrations of 30 μM or greater, and not at lower, but still embryotoxic concentrations. This does not exclude the possibility that CQ may inhibit receptor-mediated endocytosis or other forms of uptake in the rat conceptus.

Besides inhibition of VYS nutritional function, CQ embryotoxicity could result from several other effects of the drug on cell function. For instance, relatively low concentrations of CQ can inhibit DNA synthesis in certain cell culture systems [46]. According to Krogstad and Schlesinger [9], inhibition of DNA polymerase requires CQ concentrations of 1–2 mM. Thus, the CQ concentrations that we have estimated in conceptal tissues appear high enough to inhibit DNA synthesis in the VYS, but most likely not the embryo proper. Although we assume that most of the drug is localized in the vacuolar system, we cannot currently estimate CQ concentrations in the nucleus and so can only speculate that CQ may inhibit DNA synthesis in the VYSs of exposed conceptuses. Despite this possibility, the most likely explanantion for the *in vitro* embryotoxicity of CQ which we have obseved is that CQ alters the lysosomal processing for proteins by the VYS, resulting in a diminished amino acid supply for both the VYS and embryo.

In conclusion, the current data show that CQ-induced embryotoxicity is associated with the accumulation of high concentrations of the drug in conceptal tissues and changes in the protein content and activity of lysosomal cysteine proteinases in these tissues. In contrast, inhibition of VYS fluid-phase pinocytosis by CQ cannot be detected until conceptal viability begins to be affected (30 μM CQ). The increased enzyme activity we observed occurs as a general response of conceptuses to lysosomotropic amines, and can be detected after exposure to nonembryotoxic concentrations of these compounds. This suggests that increased lysosomal cysteine proteinase activity is a compensatory response of organogenesis-stage rat conceptuses to such compounds, and measurement of this activity may be useful as an indicator of conceptal exposure to agents that alter lysosomal function.

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