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Effect of 6-hydroxydopamine on bovine adrenal chromaffin cells in culture

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1 The effect of 6-hydroxydopamine on the catecholamine content and cell morphology of bovine adrenal chromaffin cells in culture was investigated.

2 6-Hydroxydopamine markedly reduced the catecholamine content of the cultured chromaffin cells after 6 and 24 h exposure. The effect was dose-related, with half-maximal depletion occurring at 2.4×10^{-5} M

3 Cells exposed to 6-hydroxydopamine for 3 h and then to normal medium without the drug for 3 h more showed the same degree of toxicity as cells exposed to the drug for the entire 6 h.

4 Ascorbate at high concentrations also exhibited toxicity toward chromaffin cells between 6 and 24 h of exposure.

5 6-Hydroxydopamine produced marked changes in cell morphology. At 1 h the cells appeared normal, at 3 h the processes were markedly shortened, and at 6 h they were completely retracted. On exposure for 24 h there were gross morphological changes and most cells were detached and free-floating in the medium.

6 The toxicity of 6-hydroxydopamine in bovine adrenal chromaffin cells is discussed.

Introduction

Tranzer & Thoenen (1967, 1968) have shown that 6-hydroxydopamine causes selective destruction of adrenergic nerve endings without affecting the cell bodies of the sympathetic ganglion or the chromaffin cells of the adrenal medulla. One explanation for the observation that 6-hydroxydopamine has a particular selectivity for the adrenergic nerve terminals is that they accumulate toxic levels of this agent through an imipramine-sensitive 6-hydroxydopamine uptake system into the nerve terminals (Stone, Porter, Stavorski, Ludden & Totaro, 1964). This uptake system may be lacking in chromaffin cells of the adrenal medulla and the cell bodies of the sympathetic ganglia. An alternative suggestion is that, *in vivo*, 6-hydroxydopamine does not have access to the adrenal medulla or to sympathetic ganglia, hence toxic concentrations of this agent may not be reached in these tissues. This lack of access may be due to permeability barriers or a lower rate of perfusion of these tissues as compared to sympathetic nerve terminals. Difficulty in reaching the tissues at sufficiently high concentrations may indeed be true when one considers the effect of 6-hydroxydopamine on catecholaminergic neurones in the central nervous system. Since 6-hydroxydopamine does not cross the blood-brain barrier, this agent has to be injected intraventricularly to demonstrate its effects. When injected in this manner, 6-hydroxydopamine depletes catecholamines (CA) from the central adrenergic neurones, and in contrast to findings in sympathetic nerve terminals of peripheral organs (Haeusler, Haefely & Thoenen, 1969), there is no evidence of CA recovery (Bloom, Grappetti, Revuelta & Costa, 1969; Uretsky & Iversen, 1969). This observation would suggest that, in the brain, 6-hydroxydopamine destroys not only the nerve terminals, but also the cell bodies.

To address this problem, we have studied 6-hydroxydopamine toxicity in isolated cultured bovine adrenal chromaffin cells, where access of the drug should not be a factor, and have found that 6-hydroxydopamine is toxic to adrenal chromaffin cells in a dose- and time-related manner. Moreover, ascorbate used as an antioxidant was also found to have a certain degree of toxicity towards chromaffin cells with time.

Methods

Cell cultures

Chromaffin cells were plated on collagen-coated plastic (Corning) dishes (7.5×10^5 cells/35 mm dish), as previously described (Trifaró & Lee, 1980) in Dulbecco's modified Eagle's medium (DMEM, Gibco), containing foetal calf serum (10%), ascorbic acid (0.1 mM), glucose (5.6×10^{-3} M), HEPES buffer (3.6 $\mu g\,ml^{-1}$), and the following antibiotics: penicillin (100 $\mu g\,ml^{-1}$), streptomycin (100 $\mu g\,ml^{-1}$), tetracycline (5 $\mu g\,ml^{-1}$), gentamycin (10 $\mu g\,ml^{-1}$) and mycostatin (25 units ml^{-1}). The medium also contained 10^{-5} M 5-fluorodeoxyuridine and 10^{-5} M cytosine arabinoside to prevent fibroblast proliferation by inhibiting cell division. The culture dishes were incubated at 37°C in a humidified incubator (National) under a CO_2 + air (5:95) atmosphere. The culture medium was changed every 2–3 days, and cultures were also inspected by phase contrast optics.

6-Hydroxydopamine toxicity dose-response curve

6-Hydroxydopamine toxicity dose-response curves were determined on 10-day-old chromaffin cell cultures. Culture dishes were removed from the incubator, DMEM was removed, and the cells were incubated with 2 ml of the same medium for 15 min. Following this preincubation the plates were divided into 8 groups of 9 culture dishes per group. These groups consisted of two controls: one was normal DMEM, and the other was DMEM with ascorbate (5.7×10^{-3} M). The remaining 6 groups contained 6-hydroxydopamine concentrations of 4.9×10^{-6}, 2.4×10^{-5}, 4.9×10^{-5}, 2.4×10^{-4}, 4.9×10^{-4}, and 9.7×10^{-4} M in DMEM containing 5.7×10^{-3} M ascorbate. The volume for all plates was 1 ml. Three culture dishes from each of the group were removed at the end of each of the exposure periods of 1, 6, and 24 h and assayed for catecholamine (CA) content as described below. All incubations were at 37°C under a 95% O_2 and 5% CO_2 atmosphere.

Reversibility of 6-hydroxydopamine toxicity

Four groups of culture dishes were used to test the reversibility of 6-hydroxydopamine toxicity. These consisted of DMEM control, DMEM with ascorbate (1.7×10^{-3} M) for the 6 h exposure, 9.7×10^{-4} M 6-hydroxydopamine in DMEM containing ascorbate (1.7×10^{-3} M) for the 6 h exposure, and 9.7×10^{-4} M 6-hydroxydopamine in DMEM also containing ascorbate (1.7×10^{-3} M) for 3 h, followed by DMEM-ascorbate (1.7×10^{-3} M) for 3 h. The volume for all groups was 1 ml and incubation was at 37°C under a 95% O_2 and 5% CO_2 atmosphere. At the end of the 6 h period, the culture dishes were examined by phase-contrast optics, the cells scraped, and the CA contents determined.

Microscopic examination

At appropriate times, all culture dishes were examined by phase-contrast optics to determine the effects of ascorbate and 6-hydroxydopamine on cell morphology and viability. We also monitored the effects of 6-hydroxydopamine on a single cell during an 8 h exposure period to 9.7×10^{-4} M 6-hydroxydopamine in 1 ml of DMEM containing 1.7×10^{-3} M ascorbate at room temperature.

Catecholamine assay

Total cell CA content was used as one index of chromaffin cell viability. After harvesting the cells in 1 ml of 0.4 N perchloric acid, the CA content of each culture dish was determined by the method of Anton & Sayre (1982). CA values are expressed in noradrenaline equivalents (μg) per culture dish (7.5×10^5 cells per dish).

Statistics

All values are expressed as mean ± s.e. of at least 3 observations; s.e. of less than 5% are not shown.

Results

Effect of 6-hydroxydopamine on the catecholamine content of cultured chromaffin cells

One hour exposure During 1 h exposure of chromaffin cell cultures neither ascorbate 5.7×10^{-3} M nor the various doses of 6-hydroxydopamine (4.9×10^{-6} to 9.7×10^{-4} M) used had any observable toxic effects as evidenced by either microscopic examination of the total cell CA content.

Six hour exposure As the cells were exposed for longer periods of time, 6-hydroxydopamine markedly reduced the CA contents and produced changes in the cell morphology. Figure 1a shows the effect of various doses of 6-hydroxydopamine on the cell CA content after 6 h. The effect of 6-hydroxydopamine was dose-related, with half-maximal depletion occurring at about 2.4×10^{-5} M and maximal depletion at 2.4×10^{-4} M. At this time ascorbate alone did not have any effect on the CA content of the chromaffin cells.

Twenty- four hour exposure When the exposure of the cells to 6-hydroxydopamine was continued for up

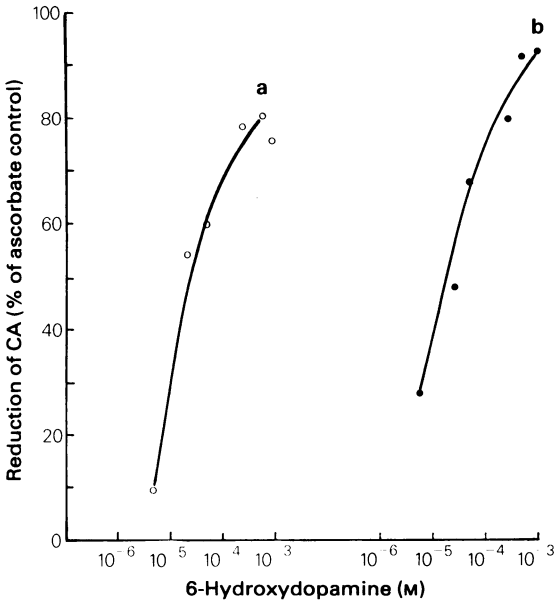

Figure 1 Effect of 6-hydroxydopamine on catecholamine (CA) content of bovine chromaffin cells in culture. Log dose-responde curves of 6-hydroxydopamine (M) versus percentage reduction of chromaffin cell CA content as compared to ascorbate (5.7×10^{-3} M) controls. Curve (a) is at 6 h of exposure and curve (b) is at 24 h of exposure. CA content of the ascorbate controls in (a) and (b) were 25.06 ± 0.44 and 9.4 ± 0.1 μg per three plates, respectively.

to 24 h, the CA content was also markedly reduced in a dose-dependent manner. It should be pointed out, however, that, at 24 h, ascorbate itself exhibited toxicity, as shown by a 68% decline in the CA content of the cells exposed to ascorbate alone (5.7×10^{-3} M. Even though ascorbate exhibited toxicity at this time, a dose-response curve to 6-hydroxydopamine was still obtainable (Figure 1b).

Test of the irreversibility of the 6-hydroxydopamine toxicity

An experimental protocol was devised to determine whether the toxicity of 6-hydroxydopamine toward adrenal chromaffin cells is reversible if the agent is removed after a 3 h exposure and replaced by normal medium. Results are shown in Figure 2. A 3 h exposure to 6-hydroxydopamine initiates an irreversible toxic process, since the depletion of CA was the same at 3 h as at 6 h. Also of note from Figure 3 is that 1.7×10^{-3} M ascorbate is not toxic at 6 h of incubation, and that 6-hydroxydopamine gave the same degree of toxicity when DMEM contained 1.7×10^{-3} M ascorbate as when DMEM contained 5.7×10^{-3} M ascorbate during the dose-response curves. As in the dose-response curve determinations, these observations were amplified by microscopic examination of all the dishes. The DMEM control and the ascorbate in DMEM control were normal, healthy-appearing cultures, and the adrenal chromaffin cells had normal processes. The 6-hydroxydopamine-exposed cultures showed evidence of cell death, and the processes on the adrenal chromaffin cells had retracted.

Effect of 6-hydroxydopamine on the morphology of chromaffin cells in culture

At 1 h exposure neither ascorbate nor 6-hydroxydopamine produced any morphological changes. At 6 h, cells exposed to ascorbate alone appeared healthy and the chromaffin cell processes normal, with no obvious morphological changes. In the 6-hydroxydopamine-exposed plates the adrenal chromaffin cells had retracted their process, and some cells were rounded. At higher concentrations these effects were much more pronounced, and some cells were freely floating in the medium. Figure 3 shows the effect of 6-hydroxydopamine on a single cell that had developed processes. The changes in this cell were followed for 8 h at room temperature while the culture dish was fixed on the microscope stage. At 1 h, the cell looked normal and the processes intact. At 3 h the processes were markedly shortened, and at 6 h they were completely retracted. As mentioned earlier, at this time the cells have lost nearly 80% of their catecholamine content.

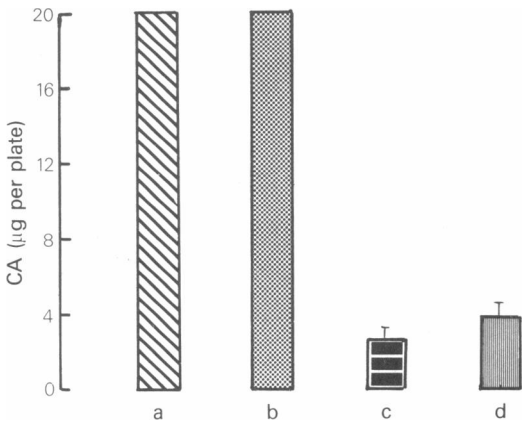

Figure 2 Irreversibility of 6-hydroxydopamine toxicity toward bovine chromaffin cells in culture. (a) DMEM control; (b) ascorbate (1.7×10^{-3} M) in DMEM for 6 h; (c) 6-hydroxydopamine (9.7×10^{-4} M) in DMEM containing ascorbate (1.7×10^{-3} M) for 6 h (d) 6-hydroxydopamine (9.7×10^{4} M) in DMEM containing ascorbate (1.7×10^{-3} M) for 3 h, followed by incubation in regular DMEM for an additional 3 h.

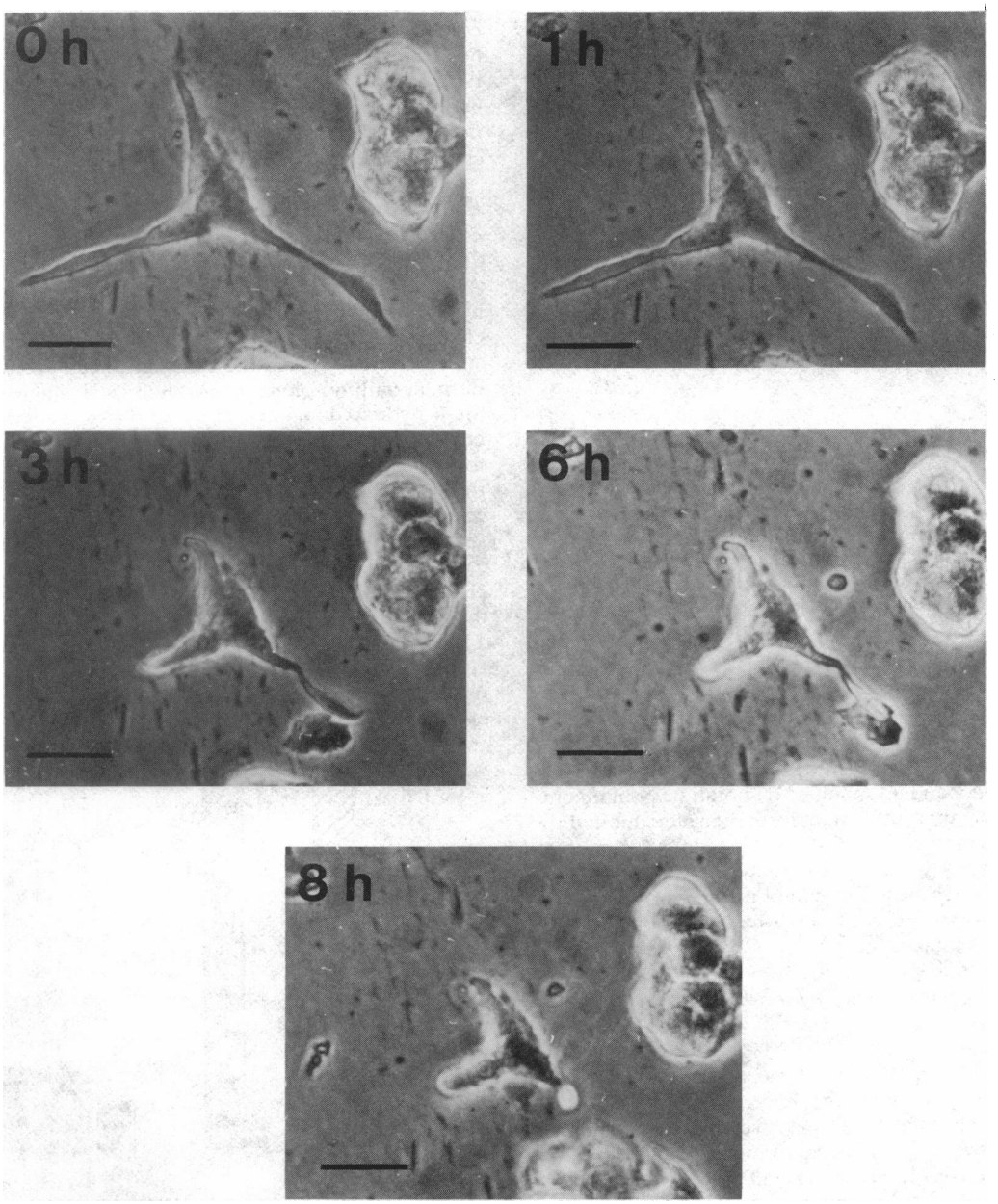

Figure 3 Chromaffin cell grown for 10 days on a collagen-coated culture dish and incubated for 8 h at room temperature with DMEM containing 9.7×10^{-4} M 6-hydroxydopamine and 1.7×10^{-3} M ascorbate. The cell was examined by phase-contrast optics, and at the intervals indicated above (0, 1, 3, 6 and 8 h), photographs were taken with a 35-mm Kodak Tri-X pan film (ASA 400). The horizontal bars represent 50 μm.

On 24 h exposure there were gross morphological changes. The few cells which remained adhered to the plate were rounded. The majority of them were detached and free-floating in the medium.

Discussion

The dose-response curves of 6-hydroxydopamine concentration vs. CA content of adrenal chromaffin cells, the experiments showing lack of reversibility of 6-hydroxydopamine toxicity, and the microscopic studies demonstrating the progressive adrenal chromaffin cell deterioration in the presence of 6-hydroxydopamine all clearly demonstrate that 6-hydroxydopamine is toxic to adrenal chromaffin cells. Ascorbate (5.7×10^{-3} M) was found to be toxic at some point between 6 and 24 h of exposure, but at a lower concentration (1.7×10^{-3} M) it was not toxic.

The 6-hydroxydopamine toxicity is distinct from the ascorbate toxicity, since the 6-hydroxydopamine toxicity manifests itself in a dose-related manner at 6 h of exposure, a time when ascorbate had not demonstrated any toxicity. Also at 24 h of exposure to 6-hydroxydopamine with ascorbate (5.7×10^{-3} M), a dose-response curve for 6-hydroxydopamine is still abtainable despite the toxicity demonstrated by the ascorbate. Of interest is the fact that the dose-response curves for 6 and 24 h of exposure to 6-hydroxydopamine are very similar. One additional point which distinguishes the 6-hydroxydopamine toxicity from the ascorbate toxicity at 6 h is that the same degree of toxicity is obtained in the presence of 5.7×10^{-3} M or 1.7×10^{-3} M ascorbate. It was also shown that after 3 h exposure of chromaffin cells to 6-hydroxydopamine the toxicity is irreversible. It is quite possible that this exposure time may be much shorter than 3 h, since 6-hydroxydopamine, like dopamine, may be readily taken up inside the chromaffin cells and converted rapidly to a toxic product to produce cell destruction. Irrespective of the mechanism involved in 6-hydroxydopamine toxicity, it would seem that once the toxic process is started it is virtually irreversible.

The discrepancy between the studies demonstrating the lack of toxicity for the adrenal medulla *in vivo* *vs.* the present studies demonstrating toxicity for chromaffin cells in cell culture may possibly be explained by two major differences between the two situations (*in vivo vs.* cell culture). One is the question of access of 6-hydroxydopamine to the adrenal chromaffin cells, which may be subdivided into two areas: the possibility of permeability barriers to 6-hydroxydopamine in the adrenal medulla *in vivo*, or, secondly, lack of access due to a lower rate of perfusion of the adrenal medulla as compared to adrenergically innervated peripheral organs, leading to low concentrations of 6-hydroxydopamine reaching the adrenal medulla. Obviously, the question of access has been eliminated in cell culture.

An alternative explanation also arises from the differences in chromaffin cells *in vivo* and in cell culture. *In vivo* chromaffin cells lack processes, but in cell culture they develop nervelike processes. *In vivo* chromaffin cells could have uptake processes lacking in the adrenal medulla, but once chromaffin cells have been cultured and developed nervelike processes, they could at the same time gain an uptake system for 6-hydroxydopamine. However, it should be pointed out that a high affinity uptake mechanism for noradrenaline has been demonstrated in 2-day-old cultures where process outgrowth was apparent only in a few chromaffin cells (Kenigsberg & Trifaró (1980).

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