Vol. 88, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 13, 1979 Pages 1105-1110

EFFECT OF 20,25-DIAZACHOLESTEROL ON VIABILITY AND STEROID SYNTHESIS CAPABILITY OF CULTURED CHICK EMBRYO PECTORAL MUSCLE CELLS*

E.R. Rosenblum, J.M. Malloy, I.R. McManus, J.D. Naworal, and I.M. Campbell

Department of Biological Sciences, University of Pittsburgh
Parran Hall, 130 DeSoto Street, Pittsburgh, Pennsylvania, 15261

Received April 10,1979

SUMMARY: The anticholesterolemic drug 20,25-diazacholesterol (DAC) inhibits the transfer of radioisotope from acetate to cholesterol in cultured chick embryo pectoral muscle cells. In a 12 hour parallel exposure to tracer and drug (1 μg/ml culture fluid), inhibition was complete as determined by radiogas chromatography (RGC). Drug levels in excess of 5 μg/ml caused cell lysis. RGC/mass spectrometry (selected ion monitoring mode) revealed that desmosterol did not accumulate as a result of the treatment nor was radioactivity transferred to desmosterol. Mass and radioactivity did accumulate in other steroids. The significance of these findings on DAC's mode of action as an anticholesterolemic and myotonia-inducing agent, is discussed.

20,25-Diazacholesterol (DAC) was developed originally as an anticholesterolemic drug (1). In this regard it is effective in animals and humans (1-3). It also produces myotonia in patients and animals receiving it (2,4,5)---a side effect that has precluded its continued clinical use. The DAC-induced myotonia, however, bears a close resemblance to congenital myotonic dystrophy (6) and for this reason, interest in the pharmacology of DAC has remained high.

The current consensus is that DAC inhibits the metabolism of desmosterol (cholesta-5,24-dien-3-ol), specifically inhibiting the reduction of the Δ^{24}-double bond (see references 6-9). The principal evidence for this conclusion is that desmosterol accumulates in the serum of patients and animals treated with the drug (2,3,10-14). Implied in this theory is that reduction of the Δ^{24}-double bond is a late step in cholesterol biosynthesis.

Despite its attractiveness, it is doubtful if the desmosterol-Δ^{24}-reductase theory is completely adequate. In the first place, it is uncertain that reduction of the Δ^{24}-double bond is a late step in cholesterol biosynthesis (15).

*This work was supported by grants from the National Institutes of Health (GM-25592) and the Muscular Dystrophy Association of America.

0006-291X/79/111105-06$01.00/0

Secondly, early results of the Searle group suggested that DAC had multiple sites of action in the steroid biosynthetic pathway and that steroids in addition to desmosterol accumulated (11,16,17). In accord with this, Niemiro and Fumagalli (18) observed DAC to inhibit the 7-dehydrocholesterol Δ^7-reductase of rat liver *in vitro*.

The objective of the work whose preliminary results are reported herein, was to make a complete determination of the effect DAC had on chick embryo muscle cells. Resolution of this issue was deemed crucial to further studies of how DAC produced myotonia.

MATERIALS AND METHODS

Embryonic chick pectoral muscle cells were prepared according to the method of Konigsberg *et al.*(19). Cells were plated in the medium of Coleman and Coleman (20) on collagen-coated Falcon plastic petri dishes (150 mm) at an initial density of 5×10^6 cells. Medium was changed on the 2nd and 4th days and daily thereafter. Normally, cells were confluent by the 7th day and 70-75% of the nuclei were present in myotubes. On the 7th day, cells were depleted of serum for 4½ hours and then were treated with [1-^{14}C]acetate (10 μCi/plate) and DAC (1 μg/ml, 2.6×10^{-6}M) for 12 hours. Thereafter, a chloroform/methanol extract was made of treated and control (no DAC) cells. These extracts were examined by radiogas chromatography (RGC) and RGC/mass spectrometry (RGC/MS) both directly and after methanolysis (10% methanolic HCl, 2 hrs, 70°C). Gas chromatographic columns (0.004 x 3 m) filled with 3% OV-17 were used in all RGC and RGC/MS analyses. A mixture of cholesterol, desmosterol, lanosterol, cholesta-8,24-dien-3-ol, and cholesta-7,24-dien-3-ol (the last two compounds the generous gift of Dr. A. Sanghvi) was used in conjunction with the relative retention times of Ramsey (21,22) to calibrate columns and assist with mass spectral identification of the steroids.

RESULTS

While searching for the minimum effective dose of DAC to produce an inhibition of the transfer of isotope from [1-^{14}C]-acetate to cholesterol, it was found that DAC caused cell lysis. Levels of DAC in excess of 50 μg/ml of culture fluid caused complete lysis (as judged by light microscopy) within 15 minutes. With doses of 5, 10, and 25 μg/ml, lysis was apparent in 90 minutes. No lysis occurred with levels of 1 μg/ml or less over 48 hours. A dose of 1 μg/ml was used in the work reported here; a ten-fold reduction of dose also produces complete inhibition of isotope transfer

From radiogas chromatograms of methanolysed total lipid extracts of controls and DAC-treated cells (Figure 1, extracts have been spiked with standard

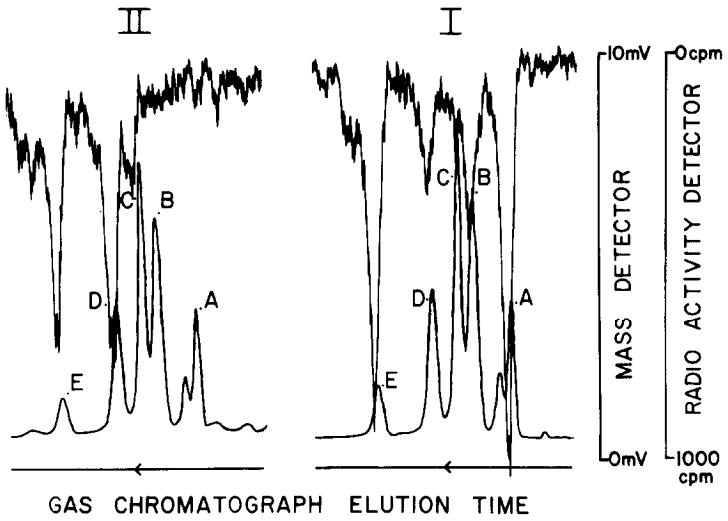

Figure 1. Section of the radiogas chromatograms obtained by spiking methanolysed nonpolar soluble extracts of control (I) and DAC-treated (II) cells with a mixture of standard steroids. Code: A = cholesterol; B = desmosterol; C = cholesta-8,24-dien-3-ol; D = cholesta-7, 24-dien-3-ol; E = lanosterol.

steroids), it was seen that: (i) [1-^{14}C]acetate was incorporated well into the cholesterol pool of control cells (I% = 0.65%); (ii) no such incorporation occurred in treated cells, (iii) other major peaks of radioactivity appear in the control cell extract in the area of the desmosterol marker (B), the cholesta-7, 24-dien-3-ol marker (D) and the lanosterol marker (E); in the extract from the treated cells the radioactivity associated with marker E remained, while that associated with markers B and D was lost and augmented respectively. These data are not compatible with DAC inhibiting the metabolism of desmosterol; such a situation would require radioactivity to accumulate in the desmosterol pool.

Further evidence that desmosterol did not accumulate in DAC-treated cells was obtained by RGC/MS analysis of un-methanolysed total lipid extracts. The mass spectrometer was focused on m/e 271, an ion that is of low abundance in cholesterol but is the base peak of desmosterol (23). There were three peaks in the m/e 271 selected ion current (SIC) profile produced by both control and treated cells (Figure 2). The first two peaks correspond respectively to cholesterol (X) and desmosterol (Y). Since the cholesterol pool in the cells is large and since in control cells the cholesterol biosynthetic rate is not

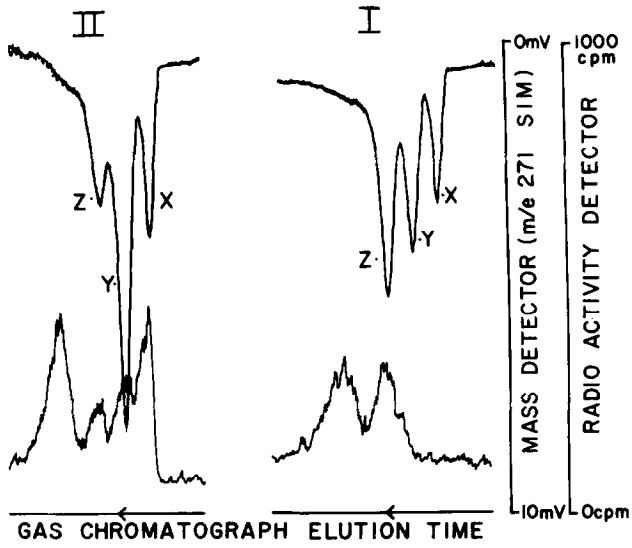

Figure 2. Section of the RGC/MS (SIM-mode) output obtained from nonpolar extracts of DAC-treated (I) and control (II) cells. Upper traces in the m/e 271 selected ion profile, lower is the radioactivity profile, Code: X = cholesterol; Y = desmosterol; Z = components with parent molecular ions m/e 398, 384 and 382.

outstandingly fast (cf. incorporation data above), it can be assumed that absolute changes in the cholesterol level in cells over the 12 hours associated with the DAC-induced inhibition of synthesis is small. Hence, the ratio of the desmosterol/cholesterol m/e 271 SIC profiles will be a measure of the amount of desmosterol in the cells. The ratio drops from a value of 2.7 in the control to a value of 1.3 in the treated cells. This is the reverse of what would be expected if DAC induced a desmosterol accumulation.

The SIC profile contained a third peak (2) in the steroid area. Its peak area increased two-fold relative to cholesterol on DAC treatment. It had the same retention time as the cholesta-7,24-dien-3-ol marker. Detailed mass spectrometric analysis (multiple scan and SIM-mode) of this area of the chromatogram of both control and treated cells indicated that materials with parent molecular ions at m/e 398, 384 and 382 coelute with the substance yielding the m/e 271 ion fragment. However, only the m/e 384 and 382 profiles show increased response in DAC-treated cells. Cholesta-7,24-dien-3-ol and cholesta-5,7,24-trien-3-ol have appropriate molecular weights and would be expected to elute in

this area; the mass spectrum of both compounds contains an intense m/e 271 ion (24-25). Both these compounds are, therefore, highly probably candidates for the maximum in the m/e 271 SIC profile. Which one(s) is (are) responsible for the radioactivity increase in the DAC-treated cells remains to be seen. RGC/MS-compatible techniques to resolve this question are currently being developed.

DISCUSSION

This work establishes that in primary chick pectoral muscle cells, DAC inhibits cholesterol biosynthesis at the level of 1 µg/ml culture fluid (2.6×10^{-6}M). Complete inhibition also occurs at 10^{-6}M. Why Boland et al. (26) failed to see any effect in the range 10^{-7}-10^{-5}M is not obvious at present.

At the 2.6×10^{-6}M level, DAC does not cause desmosterol to accumulate. Rather, desmosterol biosynthesis is inhibited by DAC and intracellular desmosterol pool decreases relative to cholesterol. The preliminary indication based on m/e 271 SIC profiles, is that cholesta-7,24-dien-3-ol and/or cholesta-5,7,24-trien-3-ol are accumulated---a finding that would be in accord with DAC's reported inhibition of a Δ^7-reductase in rat liver (18). As far as chick embryo muscle cells are concerned, therefore, the action of DAC on their steroid synthesis capability is not what it was thought to be.

Several authors have linked DAC's steroid synthesis inhibition property with its ability to produce myotonia (4,5). Our finding that DAC can cause membrane disruption indicates an alternative potential explanation for this effect.

REFERENCES

1. Counsell, R.E., Klimstra, P.D., and Ranney, R.E., (1962) J. Med. Pharm. Chem., 5, 1224-1233.
2. Winer, N., Martt, J.M., Somers, J.E., Wolcott, L., Dale, H.E., and Burns, T.W., (1965) J. Lab & Clin. Med., 66, 758-769.
3. Sachs, B.A., and Wolfman, L., (1965) Arch Intern. Med., 116, 366-372.
4. Seiler, D., Fiehn, W., and Kuhn, E., (1974) In Recent advances in myology (Bradley, W.G., Gardner-Medwin, D., and Walton, J.N., editors) Excerpta Medica, Amsterdam pp. 429-433.
5. Appel, S.H., and Roses, A.D., (1977) In Pathogenesis of human muscular dystrophies (Rowland, L.E., editor) Excerpta Medica, Amsterdam pp. 747-758.
6. Laskowski, M.B., and Dettbarn, W.D., (1977) Ann. Rev. Toxicol., 17, 387-409.

7. Peter, J.B., Campion, D.S., Dromgoole, S.H., Nagatomo, T., and Andiman, R.M., (1974) In Recent advances in myology (Bradley, W.G., Gardner-Medwin, D., and Walton, J.N., editors) Experta Medica, Amsterdam pp. 434-440.
8. Butterfield, D.A., and Watson, W.E., (1977) Membrane Biol., 32, 165-176.
9. Peter, J.B., and Campion, D.S., (1977) In Pathogenesis of hyman muscular dystrophies (Rawland, L.E. editor) Excerpta Medica, Amsterdam, pp. 739-746.
10. Thompson, M.J., Dupont, J., and Robbins, W.E., (1963) Steroids 2, 99-104.
11. Ranney, R.E., and Daskalakis, E.G., (1964) Proc. Soc. Exper. Biol. Med. 116, 999-1001.
12. Fumagalli, R., and Niemiro, R., (1964) Life Sci., 3, 555-561.
13. Burns, T.W., Dale, H.E., and Langley, P.L., (1965) Amer. J. Physiol., 209, 1227-1232.
14. Ahrens, R.A., Dupont, J., and Thompson, M.J., (1965) Proc. Soc. Exper. Biol. Med., 118, 436-438.
15. Nes, W.R., and McKean, M.L. (1977) Biochemistry of steroids and other isoprenoids. University Park Press, Baltimore. Chapter 9.
16. Ranney, R.E., and Cook, D.L., (1965) Arch. Int. Pharmacodyn., 154, 51-62.
17. Klein, P.D., Ranney, R.E., Counsell, R.E., and Szczepanik, P.A., (1963) Proc., 22, 591.
18. Niemiro, R. and Fumagalli, R., (1965) Biochim. Biophys. Acta, 98, 624-631.
19. Konigsberg, J.R., McElvain, N., Toothe, M. and Herrmann, H., (1960), J. Biophys. Biochem. Cyto., 8, 333-343.
20. Coleman, J.R., and Coleman, A.W., (1968) J. Cell Physiol., 72, Suppl. 1, 19-33.
21. Ramsey, R.B., Aexel, R.T., Jones, J.P., and Nicholas, M.J., (1971) J. Biol. Chem. 246, 6393-6400.
22. Ramsey, R.B., Aexel, R.T., Jones, J.P., and Nicholas, M.J., (1972) J. Biol. Chem. 247, 3471-3475.
23. Anonymous, (1974) In Eight peak index of mass spectra, Mass Spectrometry Data Centre, AWRE, Aldermaston, England.
24. Ener, M.A., and Frantz, I.D., (1973) J. Biol. Chem. 248, 6697-6700.
25. Scallen, J., Dean, W.J., Loughran, E.D., and Vora, V.V., (1969) J. Lipid Res. 10, 121-127.
26. Boland, R., Chyn, T., Roufa, D., Reyes, E., and Martonosi, A., (1977) Biochim. Biophys. Acta 489, 349-359.