Vol. 144, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1987 Pages 284-291

SEX MEDIATED LIPID METABOLISM IN HUMAN AORTIC SMOOTH MUSCLE CELLS

Syed M. Naseem† and Felix P. Heald*

†Johns Hopkins School of Public Health and Hygiene, Baltimore, MD 21205
and *University of Maryland, School of Medicine, Baltimore, MD 21201

Received February 26, 1987

SUMMARY: When human aortic smooth muscle cells in culture were treated with pharmacological doses of estrogen and testosterone for 48 hrs, the rate of cholesterol synthesis measured both by acetate incorporation and the 3,hydroxy 3-methylglutaryl Co enzyme A reductase (HMG-CoA) activity declined significantly as compared to control. However, the rate of cholesterol esterification increased by 132% and 45% in response to testosterone and estrogen respectively. Also, acetate incorporation into fatty acids and fatty acid synthetase enzyme activity increased by hormonal treatment but remained in the free form especially by estrogen. Testosterone treatment resulted in more esterification ($p < .025$) of fatty acid than estrogen treatment. Incubation with hormones for 48 hrs resulted in enhanced uptake of ^{14}C-labeled cholesterol along with increased accumulation of cellular cholesterol. Increased synthesis of phospholipid and triglyceride by estrogen may be responsible for excretion of cellular sterol and fat. These results indicate that sex-hormones have an important effect on the regulation of lipid metabolism in human aortic cells.

A significant aspect of atherosclerosis is the development of fatty streaks in the aorta of juveniles. The adolescent male is particularly susceptible to the formation of fatty streaks and fibrotic atheromatous plaques (1). It is generally believed, though not well documented, that increased testosterone production may be responsible for the increased incidence of atherosclerosis in men. Earlier it has been shown that there is a net decrease in serum cholesterol level at puberty in males when compared to females (2,3). Similarly, castration of male pigs increases severity of diet-induced atherosclerosis (4).

†Reprint requests to: Dr. Syed M. Naseem, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD 21701-5011.
Supported by National Institutes of Health Grant #NS16332.

0006-291X/87 $1.50

The low incidence of atherosclerosis in premenopausal women suggests that sex-linked factors may play a role in the prevention of this vascular disease (5). However, there is no documented evidence that estrogen either retards or accelerates atherogenesis. It has been suggested that exogenous administration of estrogen, both in men and women, results in a short-term increase in cardiovascular mortality (6,7). This increase in mortality is not mediated by any direct effect on the vessel wall (6). The long-term effect of estrogen on the vessel wall is unknown.

To clarify these contradictory observations, the effect of sex-hormones on lipid metabolism was studied in cultured human aortic smooth muscle cells. Since aortic cell cultures may reflect a pattern of metabolic differences that predispose to atherosclerosis, the changes in cholesterol and fat synthesis reported here may have some relevance to human disease (8).

MATERIALS AND METHODS

Materials: Hormones and co-factors for enzyme assays were purchased from the Sigma Chemical Co. (St. Louis, Mo.). Radioisotopes were obtained from New England Nuclear (Boston, Mass.). The purity of all compounds was checked by thin layer chromatography in several systems. The culture medium and all supplements were purchased from Grand Island Biological Co. (Grand Island, N.Y.).

Cell Culture: Smooth muscle cells were isolated from the aorta of a 20-year old man within one hour of death (Maryland Institute for Emergency Medical Services Systems, University of Maryland School of Medicine). Smooth muscle cells were established in culture as described by Ross (9). Optimal cellular growth was achieved by adding 30 and 10 ngm/ml epidermal and fibroblast growth factors respectively plus 1 μgm/ml fibronectin in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. The dishes were incubated at 37°C in an atmosphere of 95% air and 5% CO_2. Smooth muscle cells were distinguished from fibroblasts by their longer lag period before outgrowth from explants, their slower growth rate, and their morphological characteristics (10,11). All cells were grown in culture dishes and were used within the 6th passage for various, biochemical studies.

Hormone Solution: Before addition to the culture medium, 17-β estradiol (estrogen), testosterone, 5 α-dihydrotestosterone (DHT), fluorxymesterone were initially dissolved in ethanol and diluted with 0.02% sodium carbonate in saline as described earlier (12). The solutions were stored at -90°C. Further dilutions were made and final concentration of hormones during incubation was 10^{-7}M.

Enzyme assays and cellular cholesterol measurements: After completion of 24 or 48 hrs of incubation with hormones, the cells were washed extensively and scraped with a rubber policeman into one ml ice-chilled buffer containing 50 mM potassium phosphate (pH 7.4), 1 mM EDTA, and 30 mM nicotinamide. Cells were homogenized using a Dounce homogenizer at 4°C. Aliquots of the 900 xg supernatant were used for HMG-CoA reductase assay as described by Shapiro et

al. (13) and modified by Brown and Goldstein for fibroblast (14), and fatty acid synthetase (15) activities. Lipid was extracted by Folch's procedure (16) for cellular cholesterol measurement by the method of Allian et al. (17).

Lipid synthesis and ^{14}C-cholesterol uptake: Experimental conditions were the same as described for enzyme studies. Hormones were added at 10^{-7}M concentration for 24 or 48 hrs. Sixteen hrs before termination of the experiment, 1 μCi/ml of sodium (1,2-^{14}C) acetate per dish (spec. act. 54 mCi/mmole) was added. After removing the radioactive medium, the cells were washed four times with ice-cold phosphate buffered saline (PBS) solution, harvested with a Teflon-coated scraper and washed again. The lipid was extracted by the Folch procedure (16). The lower phase was collected, pooled, and evaporated to dryness under nitrogen. Thin layer chromatography was used for analysis and fractionation of the lipid classes by the method of Takatori et al. (18) and Sano and Privett (19). Heat-inactivated silica gel C plates were developed first with a solvent system containing petroleum ether/ethyl ether (90:10, v/v) followed by petroleum ether/ethyl ether/glacial acetic acid (80:20:0.2, v/v) to a second front 5 cm below the first. Authentic purified standards were added to visualize the separated lipids and the identified lipids were scraped, eluted, and aliquots were counted for radioactivity.

In cholesterol uptake studies, cultures were treated with hormones as described earlier and two hours before the termination of the experiment (4-^{14}C) cholesterol at 1 μCi/ml (spec. act. 56 mCi/mmole) was added. Cells were washed, scraped, and sterol was extracted. Aliquots of the extracted lipid were counted for cellular uptake of ^{14}C cholesterol by scintillation spectrometry. Each sample was counted in duplicate.

Protein determination: Protein determination was carried out according to Lowry et al. (20) using crystalline bovine serum albumin as standard.

Expression of data: Enzyme activities are expressed as the specific activity, viz., pmoles of mevalonate or fatty acid formed/mg of cellular protein/min. Statistical significance was assessed with the "t" test.

RESULTS AND DISCUSSION

When human aortic smooth muscle cells in culture were treated with pharmacological doses (10-7M) of estrogen or testosterone for 24 or 48 hrs, the specific activity of HMG-CoA reductase was increased markedly within 24 hrs of incubation. An increase in HMG-CoA reductase activity was accompanied by an increase in free and esterified cholesterol synthesis from acetate (Table 1 and Fig. 1A) by estrogen. The mechanism by which hormones, particularly testosterone, cause cholesterol esters to accumulate without suppression of HMG-CoA reductase activity is not clear. A similar estrogen effect has also been shown earlier (21,22). After 48 hrs of estrogen treatment, cholesterol ester synthesis declined slightly compared to cultures incubated for 24 hrs, perhaps due to hydrolysis and resecretion of cholesterol ester into the medium (21). In contrast to the estrogen effect, testosterone caused incorporation of 28% more radioactivity into cholesterol ester from 24 to 48

TABLE 1

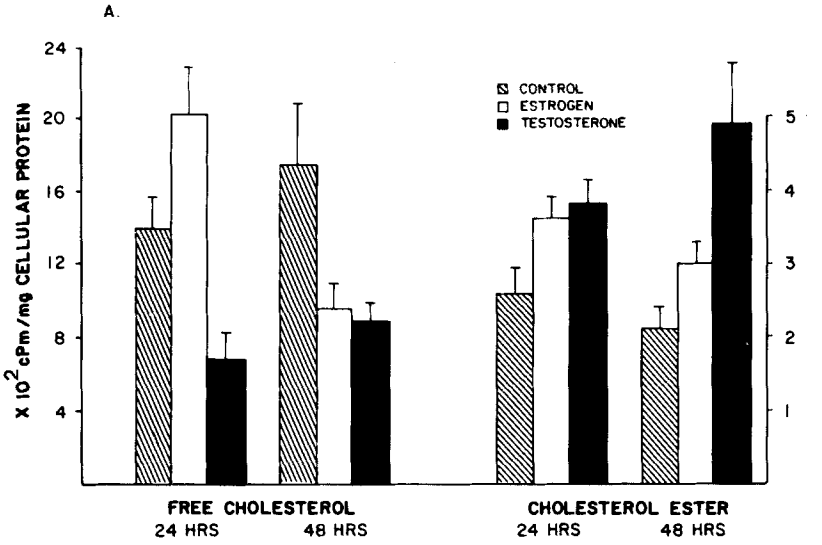
Effect of Sex-Hormones on Lipid Metabolism in Cultured Human Aortic Smooth Muscle Cells

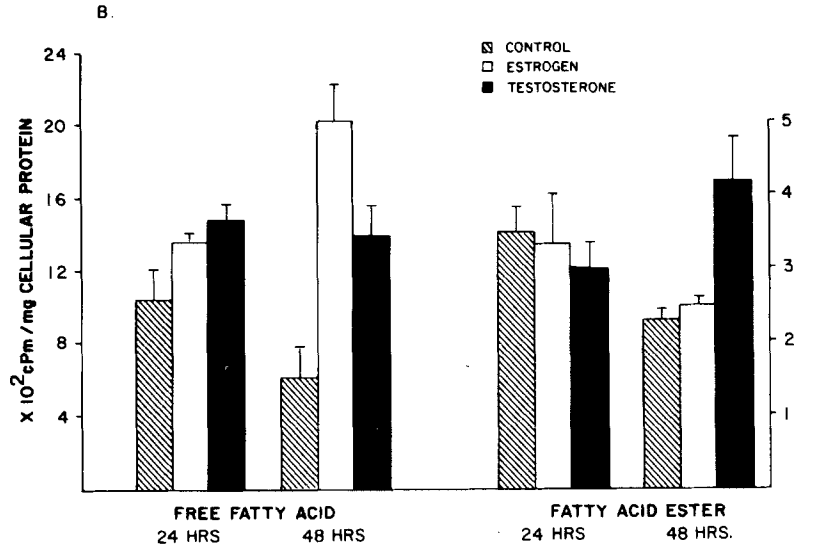
Treatment	Time hrs	HMG-CoA reductase	Fatty Acid Synthetase	Cellular cholesterol	Cholesterol uptake
		Pmoles/mg protein/ min		µgm/mg protein	Pmoles/mg protein
Control	24	40.25 ± 4.75	75.82 ± 1.79	0.829 ± 0.09	416.6 ± 34.71
	48	39.85 ± 3.25	53.93 ± 6.05	0.875 ± 0.15	428.1 ± 17.1
Estrogen	24	123.81 ± 18.20*	189.33 ± 35.92*	0.841 ± 0.29	462.4 ± 24.3
	48	62.01 ± 8.75*	120.35 ± 12.64*	1.506 ± 0.19*	540.8 ± 19.3*
Testosterone	24	101.5 ± 15.40*	148.87 ± 16.55*	0.864 ± 0.16	419.5 ± 12.0
	48	59.70 ± 3.09*+	85.13 ± 11.99*+	1.330 ± 0.26*	507.3 ± 8.1*+
Dihydro-testosterone	24	-----	-----	0.774 ± 0.16	388.2 ± 9.2
	48	-----	-----	0.853 ± 0.25	452.6 ± 26.4
Fluoxy-mesterone	24	-----	-----	0.768 ± 0.07	336.4 ± 14.6
	48	-----	-----	0.935 ± 0.17	345.9 ± 26.4

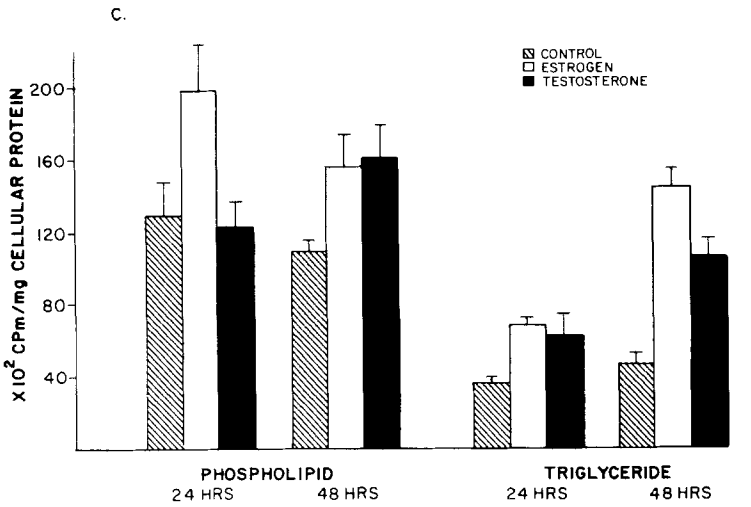
Human aortic smooth muscle cells from the stock dishes were dissociated with 0.05% trypsin-EDTA solution. After washing 2 x 10^5 cells/ml was seeded in 60 mm culture dishes with 5 ml of growth medium, consisting of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1% glutamine and growth factors, as described in "Materials and Methods". Cells were incubated for 24 hrs and then the medium was replaced with fresh 5 ml medium and hormones were added to 10^{-7}M concentration. Control cultures received vehicle only. After 24 or 48 hrs of incubation, culture dishes were washed three times with phosphate buffered saline, pH 7.4. For enzyme assays and cellular cholesterol accumulation, cellular homogenates were prepared in 50 mM potassium phosphate, 1 mM EDTA, and 30 mM nicotinamide, pH 7.4. In cholesterol uptake studies, after hormonal treatment for 24 or 48 hrs, (4-^{14}C) cholesterol at 1 µCi/ml (spec. act. 50 mCi/mmole) was added for 2 hrs at 37°C, and the tubes were placed in ice. After washing, lipid was extracted and radioactivity was measured in scintillation counter. Each value represent means ± SEM of at least three separate cultures.

* - P-value significant at least <0.01 from their respective control.
\+ - P-value significant (<0.05) at 48 hrs incubation between estrogen and testosterone treatment

hrs incubation and almost 50% more radioactivity in cholesterol ester accumulated in testosterone treated culture as compared to estrogen effect (Fig. 1A). The cholesterol ester free cholesterol ratio in testosterone rose in treated cultures which suggests that the rate of cholesterol esterification is much faster than in the control or estrogen treated cells. This increase in amount of cholesterol ester produced by testosterone may have some relevance to the increased amount of fatty streaking noted in the second decades of life


A.
CONTROL
ESTROGEN
TESTOSTERONE
X 10² cPm/mg CELLULAR PROTEIN
FREE CHOLESTEROL
24 HRS
48 HRS
CHOLESTEROL ESTER
24 HRS
48 HRS


B.
CONTROL
ESTROGEN
TESTOSTERONE
X 10² cPm/mg CELLULAR PROTEIN
FREE FATTY ACID
24 HRS
48 HRS
FATTY ACID ESTER
24 HRS
48 HRS.


C.
CONTROL
ESTROGEN
TESTOSTERONE
X 10² CPm/mg CELLULAR PROTEIN
PHOSPHOLIPID
24 HRS
48 HRS
TRIGLYCERIDE
24 HRS
48 HRS

(23-25). Fatty acid ester accumulation also increased in testosterone ($p < 0.025$) but not in estrogen treated cells (Fig. 1B). The increase accumulation of fatty acid ester in the present cell model may be similar to foam cell formation from aortic smooth muscle cells *in vivo*.

Our results show that during the first 24 hrs of incubation with estrogen, smooth muscle cells prefer initially to synthesize cholesterol as indicated by the activity of HMG-CoA reductase, even though the cholesterol is available in the incubation medium as ^{14}C-cholesterol (Table 1). However, prolonged incubation (48 hrs) with estrogen, results in an almost 50% decline in HMG-CoA reductase activity and a sharp decline in cholesterol synthesis from acetate. It is also evident that the cholesterol synthesized denovo in the first 24 hrs is not accumulated in the cells (Table 1). Incubation of smooth muscle cells for 48 hrs with testosterone or estrogen show that the cellular cholesterol pool increases and suppresses cholesterol synthesis both by inhibiting HMG-CoA reductase as well as synthesis from acetate via a feedback mechanism.

Hormonal regulation of fatty acid synthesis and cholesterol metabolism in aortic smooth muscle cells suggests the possibility that acetyl CoA, a common precursor for both HMG-CoA and fatty acid synthesis, could be channeled through the fat synthesizing pathway more efficiently when enough cholesterol accumulates in the cells or when exogenous cholesterol is available for utilization. This hypothesis is supported by the present results, since 48 hrs

---

Fig. 1. Lipid synthesis in human aortic smooth muscle cells: Conditions are same as described in Table 1. For lipid synthesis, 16 hrs before termination of the experiment, 1 μCi/ml of sodium (1,2-^{14}C) acetate (spec. act. 54 mCi/mmole) was added. After washing, lipid fractions were separated by TLC, identified with internal standards, scraped, and radioactivity was measured. Each value represents mean ± SEM of three separate plates.

P-value significant between control and estrogen-treated cells at 24 hrs: free cholesterol (<0.05), cholesterol ester (<0.01), phospholipid (<0.025), triglyceride (<0.025). At 48 hrs: free cholesterol (<0.01), cholesterol ester (<0.05), free fatty acid (<0.001), phospholipid (<0.001), triglyceride (<0.001). Comparison of control and testosterone at 24 hrs: free cholesterol (<0.001), cholesterol ester (<0.05). At 48 hrs: free cholesterol (<0.01), cholesterol ester (<0.025), free fatty acid (<0.05), fatty acid ester (<0.005), triglyceride (<0.025). Similarly, P-value is significant between estrogen- and testosterone-treated cells at 24 hrs: free cholesterol (<0.005), phospholipid (<0.01). While at 48 hrs: cholesterol ester (<0.05), free fatty acid (<0.025), fatty acid ester (<0.025), triglyceride (<0.025).

after estrogen treatment HMG-CoA reductase activity declined by almost 50% (Table 1) while cholesterol accumulation and cholesterol uptake from the incubation medium increased by 79% and 17% respectively, when incubated from 24 to 48 hrs with estrogen (Table 1). This was accompanied by a large increase in fatty acid synthetase activity and fatty acid synthesis from acetate (Fig. 1B).

It is known that removal of cholesterol is stimulated by phospholipid rich lipoprotein, particularly HDL (26,27). We have also observed that feeding an atherogenic diet to female miniature pigs increased serum cholesterol content, partially due to a greater increase in HDL cholesterol content when compared to males (28). Thus, estrogen may increase the HDL cholesterol concentration in serum as well as phospholipid and triglyceride synthesis (Fig. 1C). A cellular increase in phospholipid and triglyceride may facilitate the removal of cellular sterol and fat by increasing the fluidity of cellular membrane (29,30).

The results in Table 1 indicate that some of the alterations in lipid synthesis after testosterone treatment was also an estrogen effect, presumably mediated via aromatization. This conclusion is supported by the fact that fluoxymesterone and 5 α-dihydrotestosterone, which can not be converted to estrogen, did not mimic the effect of aromatizable androgen. Experiments reported here have also demonstrated that testosterone, a potent suppressor of free cholesterol formation from acetate, also produces a marked stimulation in the rate of esterification of cellular cholesterol. The data indicate that cholesterol synthesis and cholesterol esterification are both tightly regulated functions in human aortic smooth muscle cells.

REFERENCES

1. Valican, D. and Valican, C. (1979) Atherosclerosis 33, 201-210.
2. Levy, P.S., Hamil, P.V., Heald, F.P., and Rowland, M. (1976) Total serum cholesterol value of youth 12-17 years. Vital Health Statistics, Serial 11, #156, U.S. Dept. of Health Education and Welfare, Public Health Service, 176.
3. DeGrott, M.P.H., Marrison, J.A. (1977) Pediatrics 60, 437-443.
4. Khan, M.A., Earl, F.R., Farber, T.M., et al (1977) Exp. Mol. Pathol. 26, 63-74.
5. Tepperman, J. (1971) Metabolic and Endocrine Physiology Yearbook, Chicago, 69, 63.

6. McGill, H.C. and Stern, M.P. (1979) In Atherosclerosis Reviews (R. Paoletti and A.M. Gotto, Jr., eds.), Raven Press, New York, 4, 157-242.
7. Kolata, G. (1983) Science 220, 1137-1139.
8. Nicolson, R.J., Santerre, R.F., and Smith, S.C. (1972) Exp. Mol. Pathol. 17, 29-34.
9. Ross, R. (1971) J. Cell. Biol. 50, 172-186.
10. Rosen, L.A., Holles, T.M., and Sherman, N.G. (1974) Exp. Mol. Pathol. 20, 673-682.
11. Gimbrone, M.A., Cotram, R.S., and Folkman, J.A. (1974) J. Cell. Biol. 60, 673-684.
12. Naseem, S.M. and Hollander, V.P. (1973) Cancer Res. 33, 2909-2912.
13. Shapiro, D.J., Imbleum, R.L., and Rodwell, V.W. (1969) Anal. Biochem. 31, 383-390.
14. Brown, M.S., Goldstein, J.L. (1974) J. Biol. Chem. 249, 7306-7314.
15. Mackall, J.C., Student, A.K., Palakis, S.E., et al (1976) J. Biol. Chem. 251, 4662-4666.
16. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
17. Allian, E.E., Poon, L.S., Chan, C.G.S., Richmond, W., and Fu, P.C.L. (1974) Clin, Chem. 20, 470-475.
18. Takatori, T., Phillips, F.C., Shimaski, H., and Privett, O.S. (1976) Lipid 11, 272-277.
19. Sano, M. and Privett, O.S. (1979) Lipid 15, 337-341.
20. Lowry, D.H., Rosenbrough, N.J., Farr, A.L., and Randall. R.J. (1951) J. Biol. Chem. 193, 265-275.
21. Kovanan, P.T., Goldstein, J.L., and Brown, M.S. (1978) J. Biol. Chem. 253, 5126-5132.
22. Windler, E.T.T., Kovanan, P.T., Chao, Y.S., Brown, M.S., Havel, R.J., and Goldstein, J.L. (1980) J. Biol. Chem. 255, 10464-10471.
23. Smith, E.B. (1974) Adv. Lipid Res. 12, 149-153.
24. Smith, E.B. (1975) Atherosclerosis 22, 517-528.
25. Smith, E.B. and Smith, H.R. (1976) In Atherosclerosis Reviews (R. Paoletti and A.M. Gotto, Jr., eds.) Raven Press, New York, 1, 119-133.
26. Stein, O. and Stein, Y. (1973) Biochem. Biophys. Acta 326, 232-244.
27. Bailey, J.M. (1967) Lipid Metabolism in Tissue Culture Cells (G.A. Rothblat and D. Kritchevsky, eds.) Wistar Institute Press, Pa., 85.
28. Naseem, S.M., Khan, M.A., Sundram, G.S., and Heald, F.P. (1986) Lipids (In press).
29. Jain, M.K. (1975) Current Topics in Membrane and Transport 6, 1.
30. Malchoff, D.M., Marinetti, J.V., Havell, J.D., and Misler, A. (1979) Exp. Cell. Res. 118, 377-381.