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Inhibition of 5α-reductase activity in human skin by zinc and azelaic acid

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SUMMARY

The effects of zinc sulphate and azelaic acid on 5α-reductase activity in human skin were studied using an *in vitro* assay with 1,2[^{3}H]-testosterone as substrate. When added at concentrations of 3 or 9 mmol/l, zinc was a potent inhibitor of 5α-reductase activity. At high concentrations, zinc could completely inhibit the enzyme activity. Azelaic acid was also a potent inhibitor of 5α-reductase; inhibition was detectable at concentrations as low as 0·2 mmol/l and was complete at 3 mmol/l. An additive effect of the two inhibitors was observed. Vitamin B6 potentiated the inhibitory effect of zinc, but not of azelaic acid, suggesting that two different mechanisms are involved. When the three substances were added together at very low concentrations which had been shown to be ineffective alone, 90% inhibition of 5α-reductase activity was obtained. If this inhibition is confirmed *in vivo*, zinc sulphate combined with azelaic acid could be an effective agent in the treatment of androgen related pathology of human skin.

Skin is a target tissue for androgens and, as a result, increased androgen activity is accompanied by a well-defined skin pathology including hyperseborrhoea, acne and hirsutism or alopecia. In skin, as in many other target tissues, the reduction of testosterone to dihydro-testosterone (DHT) by the enzyme 5α-reductase is the most important of the enzymatic processes involved in androgen activity; indeed DHT is a more potent androgen than is testosterone, due to its greater affinity for the androgen receptor. The active DHT is formed at the target cell site and the enzyme 5α-reductase, therefore, acts as an amplifier of the androgen signal.[1] DHT is generally considered responsible for the stimulation of the sebaceous gland[2] and increased local formation of DHT has been documented in acne[3] and in hirsutism.[4] Any product capable of limiting local DHT production could represent, therefore, a potential therapeutic agent. The trace element zinc has been used in dermatology from the times of the Ancient Egyptians; only more recently has it been shown to reduce sebum secretion.[5] In addition it is involved in numerous enzyme systems[6] and has been reported to play a regulatory role in testosterone

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metabolism in the human prostate gland.[7] We have previously shown that zinc can inhibit, *in vitro*, the 5α-reductase of human skin.[8] Also, topical application of azelaic acid has been reported to have beneficial effects on acne vulgaris.[9] In the present study we have examined the *in vitro* effects of zinc and azelaic acid on testosterone metabolism and in particular, on 5α-reductase activity.

METHODS

Materials

1,2[^{3}H]-Testosterone (specific activity 60 mCi/mmol), [^{14}C]-testosterone (specific activity 60 mCi/mmol), [^{14}C]-dihydrotestosterone (specific activity 58 mCi/mmol) and [^{14}C]-Δ4-androstenedione (specific activity 57 mCi/mmol) were obtained from Amersham, France and purified on celite columns before use. [^{14}C]-androstanediols (3α and 3β Adiols) were prepared by sodium borohydrate reduction of [^{14}C]-DHT as previously described.[10] Unlabelled steroids were purchased from Sigma (St. Louis, MO, U.S.A) and crystallized in methanol before use. Stock solutions (10 mmol/l) were prepared in ethanol and stored at 4°C. NADPH was obtained from Boehringer Mannheim, France S.A. (Meylan) and diluted in buffer before use. All solvents and reagents were of analytical grade. Zinc sulphate, azelaic acid and pyridoxine (vitamin B6) were kindly provided by the Laboratoires Bailleul.

Tissue preparation

Foreskins from normal children (2–3 months old) undergoing circumcision were obtained, with informed consent from the parents, snap frozen in liquid nitrogen and stored at −80°C. They were then homogenized with a Polytron homogenizer (five 10s strokes) in ice-cold Krebs-Ringer phosphate buffer (120 mmol/l NaCl, 4·8 mmol/l KCl, 2·6 mmol/l $CaCl_2$, 1·2 mmol/l $MgSO_4$), pH 6·4 and used as a source of 5α-reductase.

Enzymatic assays

The assay for 5α-reductase activity in human skin homogenates has been reported previously.[11] Increasing concentrations of [^{3}H]-testosterone (9, 10, 15, 20, 30, 50, 100 and 500 nmol/l) were evaporated in glass tubes and NADPH (2·4 mmol/l) diluted in buffer was added; the reaction was initiated by the addition of an amount of homogenate corresponding to 10 mg of tissue and the volume adjusted to 1 ml with buffer. Incubations were carried out in a Dubnoff metabolic incubator at 37°C for periods of 5 to 30 min and stopped by the rapid addition of 10 ml of ethyl acetate/cyclohexane 1:1 (v/v). After addition of [^{14}C]-steroids to monitor recovery and unlabelled tracers for easy visualization, the steroids were extracted and evaporated to dryness. Tubes without homogenates, but containing the same amount of substrate, buffer and co-factors were always incubated in parallel to determine blank values. The metabolites were separated by thin-layer chromatography on silica gel, in chloroform/methanol 97·5:2·5 (v/v). Testosterone and Δ4-androstenedione were visualized under UV light (240 nm) and 5α-reduced steroids using iodine vapour. Steroids were scraped from the plate and eluted in ethanol and acetone. 3α- and 3β-adiols, not separated in this system, were eluted together. Samples were counted in a Packard 300 C liquid scintillation spectrometer with an efficiency of 68% for [^{14}C] and 30% for [^{3}H]. After correction for recovery and deduction of blank values, 5α-reductase activity was expressed as fmol (DHT + Adiols)/h/mg of tissue.

Effect of zinc and azelaic acid on 5α-reductase activity

Zinc sulphate was added to the incubation tubes at final concentrations of 0·5, 1·5, 3, 9 or

15 mmol/l diluted in 100 μl buffer, and the assay was performed as described above. Azelaic acid was added at final concentrations of 0·1, 0·2, 0·5 or 3 mmol/l diluted in 10 μl ethanol. The same amount of ethanol was also added to the control tubes. In some experiments, pyridoxin (vitamin B6) at a final concentration of 0·025% was also added to the incubation medium. These compounds were added separately or together in various concentrations in order to determine the minimal concentrations which gave a maximal inhibitory effect.

RESULTS

Preliminary experiments have shown that incubation for 5 min gave good estimates of the initial velocity of the reaction for substrate concentrations as low as 10 nmol/l (data not shown). We have used 20 nmol/l testosterone and 5 min incubations to study the effects of zinc and azelaic acid on 5α-reductase activity.

Effect of zinc and azelaic acid on 5α-reductase activity

The effects of increasing concentrations of zinc sulphate (1·5 to 15 mmol/l) and azelaic acid (0·1 to 3 mmol/l) on 5α-reductase activity were studied separately and the results are shown in Figure 1. In both cases, there was a dose-dependent inhibition of the enzyme activity; 98% inhibition was observed with 15 mmol/l $ZnSO_4$ and with 3 mmol/l azelaic acid. When the two compounds were added together to the incubation medium at concentrations expected to give 50–60% inhibition (3 mmol/l and 0·5 mmol/l, respectively), 95% inhibition was observed (data not shown).

Effect of vitamin B6 on zinc and azelaic acid inhibition of 5α-reductase activity

The addition of vitamin B6 (0·025%) to zinc sulphate (1·5 or 3 mmol/l) resulted in a two-fold increase in the inhibition of the enzyme activity (Fig. 2a). In contrast, vitamin B6 had no effect on 5α-reductase activity when added alone or together with azelaic acid (Fig. 2b). These results suggest that zinc and azelaic acid might inhibit 5α-reductase activity through two different mechanisms.

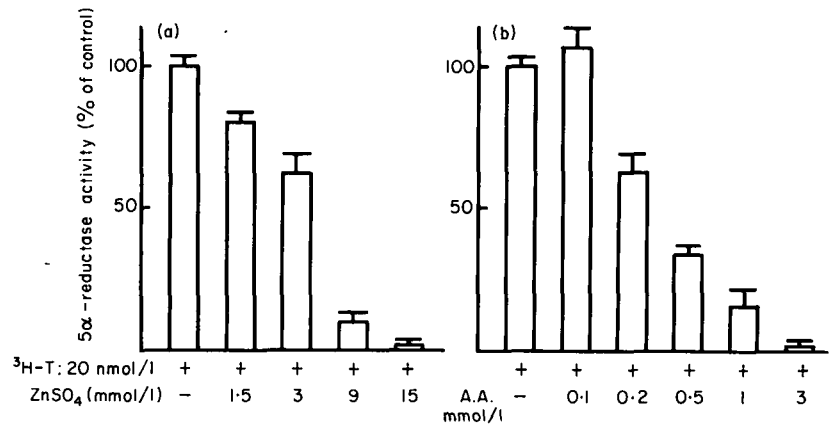

FIGURE 1. Effect of (a) $ZnSO_4$ and (b) azelaic acid (AA) on 5α-reductase activity in human skin homogenates. Results are expressed as percentages of controls without inhibitor. Values are means ± SD ($n = 6$). ^{3}H-T = ^{3}H-testosterone.

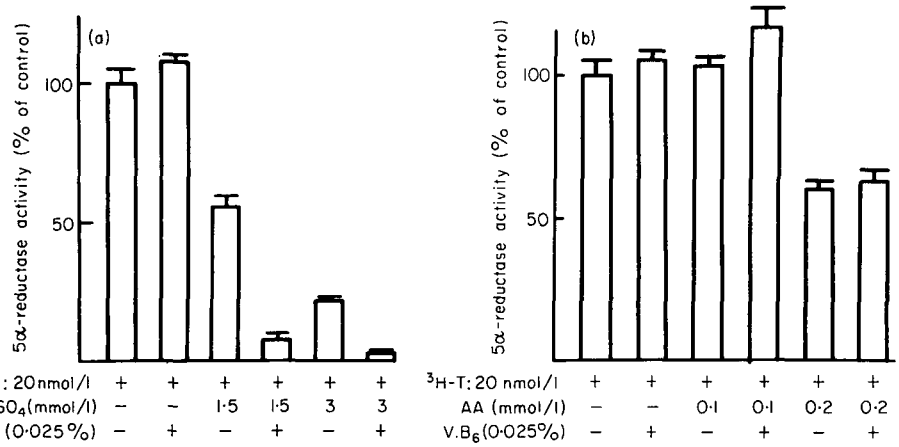

FIGURE 2. Effect of vitamin B6 (VB6) on (a) zinc sulphate and (b) azelaic acid (AA) inhibition of 5α-reductase activity in human skin homogenates. Results are expressed as percentages of controls without inhibitor. Values are means ± SD ($n = 5$). [^{3}H]-T = ^{3}H-testosterone.

Effect of simultaneous addition of zinc, azelaic and vitamin B6 at low concentrations

The additive effect of the three compounds was studied in order to determine minimal concentrations which would effectively inhibit 5α-reductase activity. From the present results, the concentrations used (0·025% vitamin B6, 0·5 mmol/l $ZnSO_4$ and 0·1 mmol/l azelaic acid) were expected to have a minimal effect, or no effect at all, on the enzyme activity. Vitamin B6 0·025% and azelaic acid at 0·1 mmol/l had no effect on the enzyme activity while $ZnSO_4$ at 0·5 mmol/l gave less than 30% inhibition (Fig. 3). In contrast, when all three compounds were added together at these concentrations, 90% inhibition of 5α-reductase activity is observed (Fig. 3).

DISCUSSION

Several previous studies have established the inhibitory action of zinc on the 5α-reductase of human prostate.[7,12,13] We have shown previously that zinc has an inhibitory effect on 5α-reductase in human skin.[8] The present study confirms this effect. In addition, since topical azelaic acid has been reported to have beneficial effects in acne vulgaris,[14] we studied the effects of azelaic acid on 5α-reductase activity. The results have shown that azelaic acid is a potent *in vitro* inhibitor of this enzyme in skin homogenates.

The use of *in vitro* assays to evaluate the local anti-androgenic action of potential 5α-reductase inhibitors has been reported previously in the study of the inhibitory effect of progesterone.[15,16] The main interest of these studies is that they enable the distinction to be made between a local and a systemic effect of the inhibitor. They are of particular value when the inhibitor can be applied topically, as is the case with zinc and azelaic acid, and therefore is less likely to exert systemic effects.

The purpose of the present study was to investigate 5α-reductase inhibition with a view to eventual application in physiological or pathological situations. As plasma testosterone levels vary from 2 nmol/l (women) to 20 nmol/l (men), it appeared pertinent to study zinc and azelaic acid inhibition of 5α-reductase activity at this range of substrate concentrations under conditions which allow a precise measurement of enzyme kinetics.

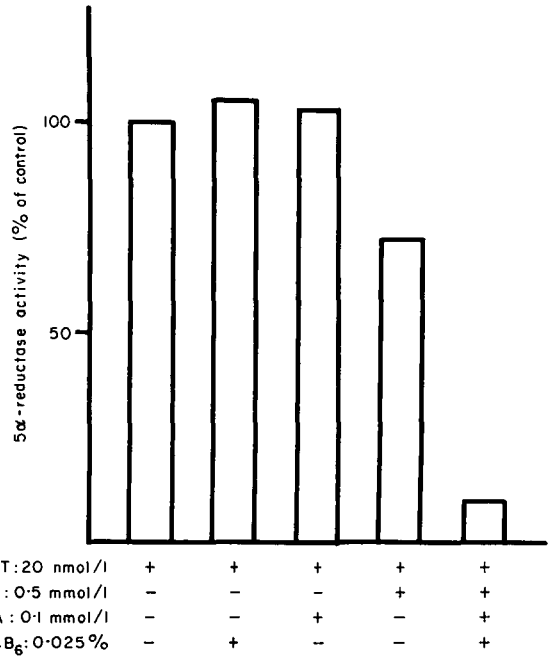

FIGURE 3. Effect of zinc sulphate, azelaic acid (AA) and vitamin B6 (VB6) alone and in combination on 5α-reductase activity in human skin homogenates. Results are expressed as percentages of controls without inhibitor. Values are means of duplicate determinations. [^{3}H]-T = ^{3}H-testosterone.

We have demonstrated a very large inhibition of 5α-reductase activity in the presence of $ZnSO_4$ at concentrations of 3 to 15 mmol/l; 98% inhibition was obtained with the highest concentration. In the human prostate, physiological concentrations of zinc are about 0·2 mmol/l and no correlation has been found between zinc concentration and 5α-reductase activity.[17] However, a biphasic effect of zinc on 5α-reductase activity in the human prostate has been reported, with potentiation at low concentrations (0·1 μmol/l) and inhibition at higher concentrations (3 to 300 mmol/l).[7] This inhibition was shown to be non-competitive relative to testosterone, but competitive relative to NADPH formation. Our preliminary studies (data not shown) seemed to indicate that zinc at low concentrations (0·5 to 3 mmol/l) competitively inhibits 5α-reductase activity while at higher concentrations (3 to 15 mmol/l) it acts as a non-competitive inhibitor. These results suggest that zinc at different concentrations may act by different mechanisms and that this metal ion interferes with different enzymes, since it also inhibits NADP reduction.

Dicarboxylic acids containing 8 to 13 carbon atoms undergo β-oxidation and have been shown to be potent inhibitors of oxydoreductases. It has been proposed that azelaic acid could competitively occupy the NADPH-binding site of 5α-reductase thus resulting in inhibition of the enzyme.[14] In our experiments, azelaic acid was a potent inhibitor of 5α-reductase activity. When zinc and azelaic acid were added together, the effect of these two inhibitors was additive suggesting that they may act by two different mechanisms.

Pyridoxin (vitamin B6) is known to interfere with fat metabolism in the skin and, therefore, to play a role, like the androgens, in the regulation of sebum excretion.[18] It improves acne lesions in adolescents[19] and is more active on topical than on systemic administration.[18] This led us to

examine the combined effects of vitamin B6 and zinc. Interestingly, whereas vitamin B6 alone had no effect on 5α-reductase activity of human skin it strongly potentiated the inhibitory effect on zinc. In contrast, vitamin B6 did not potentiate the inhibition of 5α-reductase by azelaic acid. This further supports the hypothesis that zinc and azelaic acid act by two different mechanisms.

When the three substances were tested together, 90% inhibition of the enzyme was obtained at very low concentrations which barely had any effect when tested separately.

If this inhibition is confirmed *in vivo*, a combination of these substances could provide an effective topical treatment for androgen related pathology of human skin.

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