ETHANOL - INDUCED INHIBITION OF TESTICULAR STEROIDOGENESIS IN VITRO: PREVENTION BY PYRAZOLE

DAVID H. VAN THIEL

University of Pittsburgh. School of Medicine. Department of Medicine. 1000 J Scalf Hall. Pittsburgh, PA 15261. USA

SUMMARY

Using isolated Leydig cells prepared from adult rat testes, the effects of ethanol on testicular steroidogenesis were examined in vitro. Ethanol over a dosage range of 25 to 1000 mg% markedly inhibited gonadotropin-stimulated testosterone production in a dose-dependent fashion. At little as 25 mg% (ca. 6 mM) ethanol was effective in reducing testosterone synthesis by 50%. By using 0.5 mM 4-methylpyrazole (4-MP) to block the metabolism of ethanol, the inhibitory effects of ethanol on testosterone synthesis were prevented at each of the ethanol doses used. At concentrations ≥ 1.0 mM, 4-MP, per se, inhibited testosterone production. These observations demonstrate that under acute in vitro conditions, the inhibitory effects of ethanol on testicular steroidogenesis are secondary to ethanol metabolism and 4-MP, at low doses shown not to inhibit steroidogenesis directly, prevents completely the ethanol induced inhibition of testosterone synthesis.

RESUMO

Inibição in vitro da esteroidogénese testicular induzida pelo etanol: profilaxia com pirazol

INTRODUCTION

Evidence supporting a direct adverse of ethanol upon the biosynthesis of testosterone is substantial. In animals and humans, ethanol administration has been shown to produce an immediate, dose dependent reduction in serum testosterone levels. Moreover, chronic administration of ethanol results in a marked impairment of testicular function and structure. These effects can be shown to be due to a direct effect on the testes, independent of any effects upon gonadotropin secretion by the pituitary, as ethanol inhibits the production of testosterone in male rats when ethanol-induced reductions in serum luteinizing hormone (LH) levels are prevented by systemic administration of gonadotropin.

In in vitro studies using Leydig cells from sexually mature male rats, ethanol, in a dose-dependent fashion, has been shown to inhibit testosterone synthesis. In studies addressing the possible molecular mechanisms for this inhibition, ethanol has been shown to reduce the activity of 3β-hydroxysteroid dehydrogenase/Δ4-steroid isomerase, the enzyme which is rate-limiting in testosterone synthesis from pregnenolone. More recent studies have suggested that ethanol does not inhibit testicular steroidogenesis directly, but that it must first be metabolized to acetaldehyde to exert its inhibitory effects. In addition, under in vitro conditions, NAD+ has been reported to reverse the inhibitory effects of ethanol on testosterone biosynthesis.

Using a Leydig cell preparation from the testes of sexually mature male rats, the present study directly examines the questions: 1) whether ethanol per se inhibits gonadotropin-stimulated testosterone production or 2) whether alcohol must be metabolized to exert its effects upon testosterone biosynthesis and secretion. To do this, we determined whether the potent inhibitor of alcohol dehydrogenase, 4-methylpyrazole (4-MP), prevents the inhibitory effects of ethanol on gonadotropin-stimulated testosterone biosynthesis by isolated Leydig cells maintained in tissue culture. We reason that if ethanol inhibits testosterone biosynthesis directly, the addition of 4-MP should produce no change or alternatively, may actually potentiate the inhibitory effects of ethanol by preventing its removal by metabolism. If, in contrast, it is some consequence of ethanol metabolism which is crucial in the pathogenesis of this endocrine effect of ethanol, then 4-MP, by blocking the metabolism of ethanol, should reduce or prevent its inhibitory effects.
MATERIALS AND METHODS

Animals and reagents: Male Wistar rats, 60 days of age, were used in all studies and were purchased from Charles River Breeding Laboratories, Wilmington, MA. The animals were maintained on standard rat chow (Wein Products, G and L Feeds, Greensburg, PA) and water ad libitum and were housed under a 12-hour light: dark cycle in a temperature-controlled room (25 °C) for at least 48 hours prior to use. Eagle's Minimum Essential Medium (EMEM) with Earle's salt from Gibco Laboratories, Grand Island, NY, containing 10% fetal calf serum (FCS) and 2% penicillin-streptomycin (5000 U-5000 mcg/ml) (P/S) was used as control medium. Human chorionic gonadotropin (hCG) and 4-methylpyrazole (4-MP) were obtained from Sigma, St. Louis, MO. The testosterone antibody used in the radioimmunoassay of testosterone was a gift from D. L. Loriaux, M. D., Ph. D., from the Reproductive Research Branch of the National Institutes of Child Health and Human Development, NIH, Bethesda, MD. Testosterone (1, 2, 6, 7H[N]) (94 Ci/mmol) was purchased from Amersham Corporation, Arlington Heights, IL. All other chemicals were of reagent grade.

Leydig cell preparation: Leydig cells from male Wistar rats, 60-65 days of age, were prepared using a modification of the method of Oeltman et al. Animals were sacrificed by decapitation, the testes were removed, decapsulated and quickly placed in sterile Dulbecco phosphate buffered saline, pH 7.4, which was maintained at 4 °C and was supplemented with 2% P/S. The decapsulated testes were homogenized in a loose-fitting hand-held homogenizer containing 4 ml control medium per testis. Homogenates were filtered through cheesecloth and the Leydig cell-enriched filtrate was recovered. The filtrate was centrifuged at 150 g for 15 minutes to separate intact cells from cellular debris. The resultant cell pellet was resuspended in cold EMEM containing 10% FCS and 2% P/S to achieve a final desired concentration of 3x10⁷ cells/ml. Cells were counted after treatment with trypan blue to permit visualization and quantitation of intact viable cells. This procedure results in a Leydig cell-enriched preparation containing 10-25% non-Leydig cells, the majority of which are spermatozoa.

Incubation procedures: For each experiment, the Leydig cell-enriched preparation was adjusted to a concentration of 1.5x10⁷ cells per flask. Incubations were performed in 25 ml tissue culture flasks at a total volume of 6.5 ml at 37 °C on a rocker platform under a 95% oxygen/5% carbon dioxide, water-saturated atmosphere. All experimental reagents were prepared in the supplemented EMEM medium. Human chorionic gonadotropin (hCG) in varying concentrations of 0.05 to 5.00 IU/ml was used to promote testosterone production. In our laboratory, an hCG concentration of 1.0 IU/ml has been shown to stimulate testosterone production maximally (Table 1). Therefore this dose was used in all subsequent experiments. After incubation, the cells were pelleted by centrifugation and the resultant cell pellets and aliquots of the supernatant medium were frozen for subsequent steroid analysis.

For the ethanol experiments, 1.5x10⁷ cells per flask were preincubated for 30 minutes with hCG at a concentration of 1.0 IU/ml. The hCG-pretreated cells were then incubated for one hour with ethanol in concentrations ranging from 25 to 1000 mg%. At the end of each experiment, cell viability was reassessed using trypan blue exclusion and the cells and supernatant medium were frozen for subsequent testosterone assay.

<table>
<thead>
<tr>
<th>hCG Stimulation of Testosterone Synthesis by Rat Leydig Cells in Culture</th>
<th>Testosterone (ng/10⁷ cells)</th>
<th>p Value (compared to control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.61±0.01</td>
<td></td>
</tr>
<tr>
<td>hCG 0.05 IU/ml</td>
<td>1.55±0.01</td>
<td>NSD</td>
</tr>
<tr>
<td>hCG 0.01 IU/ml</td>
<td>2.27±0.72</td>
<td>NSD</td>
</tr>
<tr>
<td>hCG 0.50 IU/ml</td>
<td>2.53±0.23</td>
<td>NSD</td>
</tr>
<tr>
<td>hCG 1.00 IU/ml</td>
<td>2.83±0.04</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>hCG 5.00 IU/ml</td>
<td>3.07±0.02</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Effect of 4-Methylpyrazole (4-MP) on hCG-Stimulated Testosterone Production by Rat Leydig Cells in Culture</th>
<th>Testosterone (ng/10⁷ cells)</th>
<th>p Value (compared to control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control hCG 1.0 IU/ml</td>
<td>3.34±0.23</td>
<td></td>
</tr>
<tr>
<td>hCG+4-MP 0.1 mM</td>
<td>3.05±0.03</td>
<td>NSD</td>
</tr>
<tr>
<td>hCG+4-MP 0.5 mM</td>
<td>3.09±0.06</td>
<td>NSD</td>
</tr>
<tr>
<td>hCG+4-MP 1.0 mM</td>
<td>1.58±0.06</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>hCG+4-MP 1.2 mM</td>
<td>1.71±0.20</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

For the 4-MP experiments, 1.5x10⁷ cells per flask, after preincubation for 30 minutes with 1.0 IU/ml hCG, were exposed for one hour to 4-MP in concentrations ranging from 0.1 to 1.2 mM. This was done to determine the maximum dose of 4-MP which would not injure the Leydig cells. For all subsequent experiments requiring 4-MP, it was used at a final concentration of 0.5 mM as this is the maximum concentration which does not alter steroid production by the Leydig cells (Table 2). In these experiments, the cells were pretreated with 1.0 IU/ml hCG and 0.05 mM 4-MP for 30 minutes prior to incubation with ethanol for one hour.

Testosterone assay: Media testosterone concentrations were determined in duplicate using the radioimmunoassay method of Neischlag and Loriaux. All samples from a given experiment were run in a single assay, thus minimizing interassay variation. The interassay variation for laboratory standards was less than 5% and the detection limit using 100 µl of media was 1.0 pg.

Statistical analysis: Analysis of variance was used for all statistical calculations and results are expressed as mean values ± standard error of the mean. Differences were considered probable significant at p value of <0.05 and significant at a value of <0.01.

RESULTS

Effects of ethanol on testicular steroidogenesis in vitro: Human chorionic gonadotropin (hCG)-stimulated testosterone production by the Leydig cell-enriched preparation is shown to be dose-dependent in Table 1. From a basal testosterone production of 1.61±0.01 ng, hCG at a dose of 1.0 IU/ml produced a 75% increase to levels of 2.83±0.04 ng (p<0.01). While raising the hCG concentration above 1.0 IU/ml caused an additional arithmetic increase in testosterone synthesis, the increment was not statistically significant.
The effect of the addition of increasing amounts of ethanol on the production of testosterone generated by cells cultures under these conditions is shown in Figure 1. Ethanol produced a dose-dependent reduction in hCG-stimulated testosterone secretion. The dose of ethanol which produced a half-maximal suppression of hCG-stimulated testosterone secretion was 25 mg%. With each increment in ethanol utilized above this level however, there was a further reduction in the amount of testosterone produced (p<0.01).

Effect of 4-methylpyrazole on testicular steroidogenesis in vitro: 4-methylpyrazole, alone, at concentrations of 0.1 and 0.5 mM, had no effect on hCG-stimulated testosterone production (Table 2). Consequently, the dose of 4-MP used in all subsequent pyrazole experiments was 0.5 mM. However, it is important to note that at the higher concentrations used in the dose-response studies (Table 2), there was a progressive inhibition in the amount of testosterone synthesized (p<0.05).

Effect of 4-methylpyrazole on ethanol-induced changes in hCG-stimulated testosterone production in vitro: 4-MP, at a dose (0.5 mM) which had effect, by itself, on the amount of testosterone produced, completely prevented the inhibitory effects of ethanol (Figure 2). After the cultures were pretreated with 4-MP, ethanol, at doses which had previously resulted in a greater than 50% suppression of testosterone production no longer was inhibitory and actually may have enhanced testosterone synthesis by the hCG-stimulated Leydig cells.

DISCUSSION

Using Leydig cells in culture, these studies demonstrate that the inhibitory effects of ethanol on testicular steroidogenesis in vitro are direct consequence of ethanol metabolism and can be prevented by inhibiting ethanol oxidation. Specifically, by using 4-MP to block the metabolism of ethanol, the inhibitory effects of ethanol on testosterone synthesis were prevented at each of the ethanol doses used.

Were ethanol a direct testicular toxin, then 4-MP, by blocking the metabolism or degradation of ethanol, should have either potentiated the inhibitory effects of ethanol or at least maintained a constant level of inhibition of testosterone synthesis. Instead, a complete reversal of the inhibitory effects of ethanol following pretreatment with 4-MP was observed.

These results support previous studies suggesting that ethanol exerts its effect on testicular steroidogenesis, at least in part, by an indirect mechanism which is in some way related to ethanol metabolism. Early studies found ethanol to be a very weak inhibitor of testicular steroidogenesis under in vitro conditions. Ethanol either had no effect on hCG-stimulated steroidogenesis in the decapsulated rat testis or inhibited it only at the extraordinarily high concentrations of 800 to 1000 mM. In studies using enzymatically-dispersed hCG-stimulated Leydig cells, Cicero et al. had to use doses of 200 mM ethanol to produce half-maximal suppression of testosterone production and Ellingboe and Varanelli required similar concentrations to produce the same response. In our studies ethanol at doses as low as 25 mg% (ca. 6 mM) was effective in reducing testosterone synthesis by 50%. The reasons for these disparate observations are unclear. There were however, obvious differences in the cell preparations used. It is possible that enzymatic treatment used by others to prepare the cells reduces the surface gonadotropin receptor content or effective gonadotropin receptor activity of Leydig cells making them less responsive to hCG. In contrast, our gentle mechanical separation may be less disruptive in this regard and thereby may account, at least in part, for the increased sensitivity of our cell preparation to the more readily apparent inhibitory effects of ethanol.

It is noteworthy that under these in vitro conditions, 4-MP directly inhibited hCG-stimulated testosterone synthesis when used at concentrations at or above 1.0 mM. In contrast to our observations, Cicero et al., under acute in vivo conditions, reported no inhibition of the increase in serum testosterone produced by hCG at any concentration of pyrazole used (0.5 mmol to 2.0 mmol/kg). In an earlier
publication, though specific data were not included, these authors alluded to attempts to use alcohol and acetaldehyde dehydrogenase inhibitors in vivo but found that such inhibitors blocked steroidogenesis by themselves.\textsuperscript{12} We were careful to determine that 4-MP, at a concentration of 0.5 mM, had no adverse upon testosterone synthesis by itself, but when used in the presence of varying inhibitory doses of ethanol completely blocked the effects of ethanol.

Some years ago, in a study by Van Thiel et al., alcohol dehydrogenase (ADH) activity was demonstrated in rat testicular homogenates. Ethanol was shown to inhibit vitamin A bioactivation to retinol in the testes by competing for the ADH required for the conversion of retinol to retinal.\textsuperscript{7} The authors suggested that ethanol may alter testicular steroidogenesis as a consequence of cofactor utilization and the resultant change in the redox state of the Leydig cell.

In addition, Ellingboe and Varanelli reported that the inhibitory effects of ethanol on gonadotropin-and cyclic AMP-stimulated Leydig cells in vitro could be reversed by the addition of NAD\textsuperscript{+} to their lysed Leydig cell preparations.\textsuperscript{11} They suggested, therefore, that ethanol was not a direct testicular toxin. Rather, they postulated that a reduction in NAD\textsuperscript{+} caused by the metabolism of ethanol, was responsible for the decrease in activity of an NAD\textsuperscript{+}-dependent enzyme involved in testicular steroidogenesis.

More recently, Gordon et al., reported that rats, chronically maintained on ethanol, had a marked deficiency in testicular 3\textbeta-hydroxysteroid dehydrogenase/-\Delta\textalpha.\textsuperscript{4, 5} Steroid isomerase activity, an enzyme which is ratelimiting in testicular steroidogenesis from pregnenolone and importantly, is NAD\textsuperscript{+}-dependent.\textsuperscript{18} They also found that NAD\textsuperscript{+} completely reversed this apparent enzyme deficiency, leading them to conclude that the effect of ethanol on testicular steroidogenesis was due to a change in the NAD\textsuperscript{+}/NADH ratio resulting in reduced 3\textbeta-hydroxysteroid dehydrogenase/-\Delta\textalpha.\textsuperscript{4, 5} Steroid isomerase activity.

In contrast to the studies of Gordon et al., Chiao et al., reported a significant reduction in 3\textbeta-hydroxysteroid dehydrogenase/-\Delta\textalpha. Steroid isomerase activity in testicular microsomal preparations from rats exposed to ethanol chronically.\textsuperscript{13} This reduction was present despite saturating levels of NAD\textsuperscript{+}. They suggested that ethanol metabolism could inhibit the 3\textbeta-hydroxysteroid dehydrogenase/-\Delta\textalpha. Steroid isomerase activity directly as well as by limiting the availability of cofactors.

In a preliminary communication, Cobb et al., using the perfused rat testis, reported that 4-MP, an inhibitor of alcohol dehydrogenase, partially blocked the inhibitory effects of ethanol on testicular steroidogenesis.\textsuperscript{18} They concluded that the metabolism of ethanol contributed, at least in part, to the toxic effects of ethanol. Similarly, Cicero et al. administered 4-MP to adult male rats prior to exposing them acutely to ethanol. These authors found that though the blood ethanol levels were higher in the pyrazole-treated animals than in controls, the inhibitory effects of ethanol on testosterone synthesis were attenuated by pyrazole pretreatment.\textsuperscript{19} They concluded therefore, that under acute in vivo conditions, the metabolism of ethanol and not ethanol, per se, inhibited testicular steroidogenesis. These latter observations, coupled with the results of the present studies, suggest that the generation of acetaldehyde, the main metabolite of ethanol, might be involved in the inhibition of testosterone synthesis observed in vitro, and possibly in vivo, after ethanol exposure.

Supporting this latter hypothesis are numerous investigations showing that acetaldehyde, under in vitro conditions, inhibits testicular steroidogenesis and is actually more potent on a molar basis than ethanol.\textsuperscript{4, 12, 18-20, 21} Badr et al. reported that acetaldehyde at concentrations of 90-360 \textmu M inhibited testicular steroidogenesis by decapsulated mouse testes.\textsuperscript{4} Similarly, using enzymatically-dispersed cell preparations of adult rat testes, Cicero et al. determined that as little as 50 \textmu M acetaldehyde, a concentration which can occur under in vivo conditions, inhibited testosterone production.\textsuperscript{12}

Using yet another model, Cobb et al., produced significant depression of hCG-stimulated testosterone production in the isolated perfused rat testis using 300 \textmu M acetaldehyde.\textsuperscript{20, 22} Finally, in examining the effects of acetaldehyde on the enzymes involved in the synthesis of testosterone from pregnenolone, Johnston et al. demonstrated that acetaldehyde acutely inhibits the activity of steroid 17, 20-lyase, the enzyme required for the removal of the side chain from 17\textalpha-hydroxyprogesterone in the production of testosterone.\textsuperscript{21}

There are obvious differences in the emphasis placed on the importance of acetaldehyde, a shift in the NAD\textsuperscript{+}/NADH ratio, or a combination of both, acting together, in mediating the in vivo and in vitro effects of ethanol and/or acetaldehyde on testicular steroidogenesis. Needless to say, the precise mechanism(s) by which ethanol and/or acetaldehyde inhibit steroidogenesis is as yet unknown and awaits further study.

Finally, we conclude from our studies that: 1) under acute in vivo conditions, ethanol is not a direct Leydig cell toxin; 2) the inhibitory effects of ethanol on testicular steroidogenesis are secondary to ethanol metabolism; 3) 4-MP, at low doses shown not to inhibit steroidogenesis, prevents completely the ethanol-induced inhibition of testosterone synthesis; and 4) 4-MP, per se, can inhibit hCG-stimulated testosterone synthesis at doses \geq 1.0mM.

\textbf{REFERENCES}


Address for reprints: David van Thiel
Chief of Gastroenterology
University of Pittsburgh
School of Medicine
1000 J Scaife Hall
Pittsburgh PA 15261. USA.