Physiological Testosterone Replacement Therapy Attenuates Fatty Streak Formation and Improves High-Density Lipoprotein Cholesterol in the Tfm Mouse
An Effect That Is Independent of the Classic Androgen Receptor

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Background—Research supports a beneficial effect of physiological testosterone on cardiovascular disease. The mechanisms by which testosterone produces these effects have yet to be elucidated. The testicular feminized (Tfm) mouse exhibits a nonfunctional androgen receptor and low circulating testosterone concentrations. We used the Tfm mouse to determine whether testosterone modulates atheroma formation via its classic signaling pathway involving the nuclear androgen receptor, conversion to 17β-estradiol, or an alternative signaling pathway.

Methods and Results—Tfm mice (n=31) and XY littermates (n=8) were separated into 5 experimental groups. Each group received saline (Tfm, n=8; XY littermates, n=8), physiological testosterone alone (Tfm, n=8), physiological testosterone in conjunction with the estrogen receptor α antagonist fulvestrant (Tfm, n=8), or physiological testosterone in conjunction with the aromatase inhibitor anastrazole (Tfm, n=7). All groups were fed a cholesterol-enriched diet for 28 weeks. Serial sections from the aortic root were examined for fatty streak formation. Blood was collected for measurement of total cholesterol, high-density lipoprotein cholesterol (HDLC), non-HDLC, testosterone, and 17β-estradiol. Physiological testosterone replacement significantly reduced fatty streak formation in Tfm mice compared with placebo-treated controls (0.37±0.07% versus 2.86±0.39%, respectively; P<0.0001). HDLC concentrations also were significantly raised in Tfm mice receiving physiological testosterone replacement compared with those receiving placebo (2.81±0.30 versus 2.08±0.09 mmol/L, respectively; P=0.05). Cotreatment with either fulvestrant or anastrazole completely abolished the improvement in HDLC.

Conclusion—Physiological testosterone replacement inhibited fatty streak formation in the Tfm mouse, an effect that was independent of the androgen receptor. The observed increase in HDLC is consistent with conversion to 17β-estradiol.

Key Words: atherosclerosis ■ cholesterol ■ hormones ■ lesion

Traditionally, testosterone has been considered detrimental to the cardiovascular system because of reports of sudden death from the abuse of high-dose anabolic steroids and the higher male incidence of coronary artery disease. Current research suggests, however, that low testosterone rather than testosterone per se is associated with coronary artery disease and that a relative deficiency in these circulating levels may lead to a proatherosclerotic environment (reviewed elsewhere).

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Beneficial effects of androgen supplementation in atherosclerosis have been described in 4 animal studies. Testosterone replacement has been demonstrated to prevent aortic cholesterol accumulation in cholesterol-fed, orchidectomized male rabbits and low-density lipoprotein (LDL) receptor knock-out mice. Similarly, treatment with the male androgen dihydroepiandrosterone also has been reported to attenuate atheroma formation in castrated cholesterol-fed rabbits. Testosterone is reported to inhibit neointimal growth in endothelial-denuded rabbit aortic rings. Contrary to these studies, 1 study demonstrated a deleterious action in male mice. Von Dehn et al reported a decrease in atheroma formation in the aortic sinus and ascending aorta after testosterone suppression by administration of the GnRH antagonist Cetrorelix, whereas testosterone treatment led to a small but significant increase in lesion size. The reason for this discrepancy is unclear; however, the majority of these
studies provide evidence for a protective role for testosterone against atherogenesis in male animal models, an action that is independent of changes in serum lipid levels. It has been speculated that the antiatherogenic effects of testosterone are mediated exclusively by its conversion to 17β-estradiol, although within the literature only 1 article directly supports this hypothesis.

Growing evidence supports the ability of sex hormones to elicit nongenomic effects (ie, responses not mediated through the classic androgen receptor [AR] but instead initiated by hypothetical androgen-specific membrane receptors). These nongenomic actions are characterized mainly by rapid onset and a lack of sensitivity toward inhibitors of transcription and protein synthesis (reviewed elsewhere). For example, testosterone has been demonstrated to elicit immediate vasodilation in a number of vascular beds through blockade of an L-type calcium channel, a mechanism that is independent of the nuclear AR. Furthermore, the ability of testosterone to regulate cellular activities independently of the classic genomic route is not confined to the vascular smooth muscle cell. Many studies have revealed the modulation of calcium channel activity by steroids in the membrane of several different cell systems. However, the involvement of these nongenomic actions in the observed antiatherosclerotic effects of testosterone is currently unknown.

The aims of the present study were to determine whether testosterone deficiency and/or the absence of a functional AR is associated with increased atherosclerosis after cholesterol feeding, to determine whether physiological testosterone replacement modulates atherosclerosis by AR-dependent or AR-independent processes, and to clarify the involvement of 17β-estradiol in any action of testosterone.

Methods

Protocol 1: Determination of the Influence of the AR and 17β-Estradiol by the Tfm Mouse

In the present study, we used the Tfm mouse, which exhibits an X-linked, single-base-pair deletion in the gene encoding the classic AR. This deletion causes a frameshift mutation in the AR mRNA, resulting in a stop codon in the amino-terminal region, leading to premature termination of AR protein synthesis. On translation, a truncated receptor protein is produced that lacks both DNA- and steroid-binding domains. Unlike the clinical situation of androgen insensitivity in men, serum levels of testosterone are reduced in the Tfm mouse compared with controls, reportedly 10-fold lower in Tfm mice compared with XY littermates (≈1.5 compared with 15 nmol/L, respectively). The lack of androgen production by the testes in Tfm mice is due primarily to loss of 17α-hydroxylase, an enzyme conduit to the steroidogenesis pathway.

Protocol 2: Determination of a Dosing Regimen to Replace Testosterone to Physiological Levels in the Tfm Mouse

To assess the impact of testosterone replacement therapy on fatty streak formation in the Tfm mouse, we first had to establish and maintain a dosing regimen to replace testosterone to physiological concentrations. Experiments were undertaken in nonsurgically prepared mice to determine the appropriate volume and frequency of 100 mg/mL testosterone esters (Sustanon100; testosterone propionate 20 mg/mL, testosterone phenylpropionate 40 mg/mL, and testosterone isocaproate 40 mg/mL, Organon Laboratories Ltd, Cambridge, UK) required to replace testosterone to physiological concentrations. The human physiological replacement dose of Sustanon100 is 3.5 mg/kg administered via a once-fortnightly intramuscular injection. Because of the increased metabolic rate of the smaller species of mammal, an adjustment of the human dose by between 5 and 15 times is commonly used. Consequently, 8-week-old Tfm mice received a single 10-μL intramuscular injection of 100 mg/mL testosterone (Sustanon100), providing a dose of 50 mg/kg (≈14 times the human dose). Mice were killed at serial intervals at 1, 2, 4, 7, 10, or 14 days after injection via a Home Office–approved schedule 1 method. The testosterone levels were measured. To ensure reproducibility of this pharmaco kinetic profile, additional Tfm mice received a second injection at day 14 and were then killed on day 15, 16, 18, or 21.

Protocol 3: Determination of the Effect of Physiological Testosterone Replacement and the Influence of the AR and 17β-Estradiol on Atheroma Formation

At 8 weeks of age, the 2 groups of mice, Tfm (n = 8) and XY littermates (n = 9), underwent either sham operation or surgical castration, respectively. Animals were allowed to recover in individual cages for 2 weeks; at 10 weeks of age, they were fed a cholesterol-enriched diet ad libitum (42% butterfat, 1.25% cholesterol, and 0.5% cholate; Special Diet Services, Essex, UK) for a period of 28 weeks.

Quantification of Fatty Streak Formation

At the end of the feeding period, all animals were killed via a Home Office–approved schedule 1 method. The heart with thoracic aorta attached was carefully dissected free from the adventitia. The basal half of the ventricles and the ascending aorta were removed, and the heart was perfused with physiological saline solution to remove any blood clots. The upper half of the heart with the aortic root attached was embedded in optical cutting temperature compound (Agar Scientific Limited, Essex, UK) and frozen at −80°C until processing for analysis of lipid accumulation in the aortic sinus. Subsequently, serial 8-μm transverse frozen sections of the aortic root with valves...
The number of animals used in the study was determined in accordance with internal and external ethical review policies. The frozen 8-μm cryosections of the aortic root were allowed to air dry onto charged slides (Surgipath Europe, Peterborough, UK) at room temperature. The sections were then rinsed with 60% isopropanol and stained for 15 minutes with Oil Red O. Slides were then rinsed in 60% isopropanol until the slides appeared colorless, washed in distilled water, and counterstained with hematoxylin for 1 minute. The slides were then washed well in tap water, rinsed in distilled water, and mounted with glycerol gelatin (Sigma, Poole, Dorset, UK). After Oil Red O staining, aortic root sections were digitally photographed. Quantification of the lipid-stained areas was performed with computer-assisted morphometry using Scion Image software (NIH-Scion Image, National Institutes of Health, Bethesda, Md). Lipid-stained areas were expressed as percentage of the intimal plus medial area, with an average value calculated for each of the 5 sections analyzed per animal.

Blood Collection

At the end of the relevant feeding or dosing period, all animals were killed via a Home Office–approved schedule 1 method. Following midline sternotomy, the rib cage was opened and whole blood was collected from the chest cavity after severance of the thoracic aorta. Whole blood for serum measurements was drawn through a 1-mL syringe (Becton Dickinson, Franklin Lakes, NJ), collected into 1.5-mL Eppendorf tubes, and centrifuged at 3000 rpm for 10 minutes; the serum was frozen at −80°C. The following analyses were made on individual serum samples.

Measurement of Total Testosterone and 17β-Estradiol

Serum quantification of total testosterone (DRG Instruments GmbH, Marburg, Germany) and 17β-estradiol (Oxford Biomedical Research, Oxford, Mich) was measured in duplicate via ELISA.

Measurement of Total and High-Density Lipoprotein Cholesterol

Serum quantification of total cholesterol (TC) and high-density lipoprotein cholesterol (HDLc) was measured in duplicate in a blinded fashion with an automated colorimetric assay by the Department of Clinical Chemistry, Royal Hallamshire Hospital, Sheffield, UK.

Calculated Measurements

Non-HDLc was calculated by subtracting HDLc from TC.

Statistical Analysis

In accordance with internal and external ethical review policies (University of Sheffield and UK Home Office, respectively), the number of animals used in the study was determined in the preliminary studies and reflects this minimum level of animal usage without compromising the statistical validity of the data. Results are expressed as mean±SEM. All data were demonstrated to be normally distributed as assessed by the Kolmogorov-Smirnov test for normality, which was confirmed using probability plots contained within the SPSS package (SPSS Inc, Chicago, Ill). Data were subsequently analyzed by 1-way ANOVA, with subsequent analysis made with Student paired or unpaired t test, as appropriate, to identity individual differences between groups. When multiple t tests were applied, to allow for type 1 errors, the probability value was modified by use of the Bonferroni correction, and the probability values displayed in the text are those obtained after the use of this correction. Significance was assumed with values of P<0.05. All procedures were carried out under the jurisdiction of the UK Home Office project license (project license number, 40/2227; personal license number, 40/7385), governed by the UK Animals Scientific Procedures Act 1986. The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Protocol 1: Determination of the Influence of AR Dysfunction and Testosterone Deficiency on Atheroma Formation

Marked fatty streak formation was observed within the aortic root of the sham-operated Tfm mice, the castrated XY littermates, and the nonsurgically prepared Tfm mice but not the nonsurgically prepared XY littermates (Figures 1 and 2). The amount of lipid deposition in the castrated XY males, sham-operated Tfm, and nonsurgically prepared Tfm mice (4.39±0.26%, 2.15±0.28%, and 3.74±0.72%, respectively) was similar although marginally significantly different in that the sham-operated Tfm mice had less lipid deposition than the nonsurgically prepared Tfm animals (Figures 1 and 2). The amount of lipid deposition in the castrated XY littermates, however, was markedly greater than that observed in nonsurgically prepared XY littermates fed the same diet for the same amount of time (4.39±0.26% versus 0.31±0.11% respectively; P<0.0001) (Figures 1 and 2).

Circulating serum levels of TC, HDLc, and non-HDLc were statistically similar in sham-operated Tfm mice compared with the nonsurgically prepared Tfm mice. However,
serum levels of TC, HDLC, and non-HDLC were markedly increased in the castrated XY males after feeding on a cholesterol-enriched diet for 28 weeks. This increase was significantly greater than that observed in the nonsurgically prepared XY littermates (15.7±4.9 versus 4.2±0.5 mmol/L, \(P<0.05\); 3.5±0.8 versus 2.2±0.4 mmol/L, \(P<0.05\); and 12.2±4.6 versus 2.1±0.2 mmol/L, \(P<0.05\), respectively).

**Protocol 2: Determination of a Dosing Regimen to Replace Testosterone to Physiological Levels in the Tfm Mouse**

Serum testosterone levels rose significantly from baseline in Tfm mice after the 10-μL intramuscular injection of 100 mg/mL testosterone at day 1 (Figure 3) and remained significantly higher throughout the remainder of the 14-day period. The mean area under the curve for this 14-day period was 19.4 nmol/L. A similar window of activity was observed after the second 10-μL intramuscular injection of 100 mg/mL testosterone at day 14, with statistically similar testosterone concentrations observed. The mean area under the curve for this second 14-day period was 18.8 nmol/L. These data revealed that a single once-fortnightly intramuscular injection of testosterone (100 mg/mL Sustanon100) was sufficient to produce consistent physiological testosterone concentrations in the Tfm mouse.

**Protocol 3: Determination of the Effect of Physiological Testosterone Replacement and the Influence of the AR and 17β-Estradiol on Atheroma Formation**

A significant reduction in fatty streak formation was observed in Tfm mice after testosterone replacement therapy compared with placebo-treated Tfm mice (0.37±0.4% versus 2.86±0.39%, respectively; \(P<0.0001\)) (Figures 1 and 4). After testosterone treatment, the level of lipid deposition within the aortic root of the Tfm mouse was almost identical to that observed in the placebo-treated XY littermates (0.37±0.4% versus 0.32±0.06%, respectively; \(P=0.9\)). The degree of lipid deposition in the Tfm mice treated with physiological testosterone replacement therapy was significantly higher after cotreatment with either the ER antagonist fulvestrant or the aromatase inhibitor anastrazole compared with Tfm mice treated with physiological testosterone replacement therapy alone (1.04±0.32% and 0.88±0.18% versus 0.32±0.06% and 0.88±0.18% respectively, \(P<0.05\)). However, both were still significantly lower than that of placebo-treated Tfm mice (1.04±0.32% and 0.88±0.18% versus 2.86±0.39%; both \(P<0.0001\)) (Figures 1 and 4).

No significant differences in serum levels of TC and non-HDLC were observed between Tfm mice receiving physiological testosterone replacement therapy or placebo. However, a significant increase in serum levels of HDL-C was observed after testosterone replacement therapy in Tfm mice.
compared with placebo-treated Tfm mice (2.81±0.30 versus 2.08±0.99 mmol/L; *P<0.05). Serum levels of HDLC in Tfm mice receiving physiological testosterone replacement therapy were comparable to those observed in the placebo-treated XY littermates (2.81±0.30 versus 3.13±0.20 mmol/L respectively; *P=0.4).

Serum levels of HDLC were lower in Tfm mice receiving physiological testosterone replacement therapy in conjunction with the aromatase inhibitor anastrazole or the ERα antagonist fulvestrant compared with Tfm mice receiving physiological testosterone replacement therapy alone, although this failed to reach statistical significance. However, when expressed more accurately as a percentage of TC, serum levels of HDLC were similarly reduced in Tfm mice receiving physiological testosterone replacement therapy in conjunction with fulvestrant or anastrazole to the levels previously reported in placebo-treated Tfm mice (Figure 5).

**Discussion**

This study has clearly demonstrated that low endogenous testosterone, be it associated with a nonfunctional AR (Tfm mouse) or a fully functional AR (castrated XY male), is associated with both fatty streak formation within the aortic root and a proatherogenic circulating lipid profile after feeding on a cholesterol-enriched diet. These observations demonstrate that fatty streak formation is a consequence of low endogenous testosterone levels in these mice and not a result of the absence of a functional AR because aortic fatty streaks were observed in surgically castrated male animals.

After cholesterol feeding, similar increases in serum TC, HDLC, and non-HDLC were observed in the sham-operated Tfm mice compared with the nonsurgically prepared Tfm mice, with the greatest proportion of cholesterol being found within the non-HDLC fraction. Similarly, castrated male mice exhibited significant increases in TC, HDLC, and non-HDLC, although this was significantly higher than that observed in both nonsurgically prepared males and sham-operated Tfm mice. However, despite these marked differences in the circulating lipid profile in the castrated male littermates compared with other experimental groups, the degree of fatty streak formation in these animals was not markedly elevated, also suggesting that the absence of testosterone rather than an abnormal lipid profile was the primary cause of the observed lipid deposition. However, the fact that marked differences in the circulating lipid profile were observed in the castrated male animals compared with the sham-operated Tfm mice suggests that AR-dependent processes closely regulate cholesterol metabolism.

These data provide evidence that low serum testosterone is linked to increased fatty streak formation. These data are in agreement with a number of animal studies that describe a deleterious relationship between aortic atherosclerosis and low endogenous testosterone levels in male animal models of atherosclerosis. Alexandersen et al.⁶ used 100 sexually mature

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**Figure 4.** Bar chart showing lipid deposition within the aortic root (expressed as a percentage of the medial area of 5 sections per animal) in placebo-treated Tfm mice (n=8), placebo-treated XY littermates (n=8), Tfm mice receiving physiological testosterone replacement with Sustanon100 (S100; n=8), Tfm mice receiving physiological testosterone replacement with S100 in conjunction with fulvestrant (n=8), and Tfm mice receiving physiological testosterone replacement with S100 in conjunction with anastrazole (n=7) after feeding on a cholesterol-enriched diet for 28 weeks. *P<0.05 vs indicated group; †P<0.0001 vs placebo-treated Tfm mice via Student unpaired t test.

**Figure 5.** Bar chart showing serum concentrations of HDLC as a percentage of TC in placebo-treated Tfm mice (n=8), Tfm mice receiving physiological testosterone replacement with Sustanon100 (S100; n=8), Tfm mice receiving physiological testosterone replacement with S100 in conjunction with fulvestrant (n=8), and Tfm mice receiving physiological testosterone replacement with S100 in conjunction with anastrazole (n=7) after feeding on a cholesterol-enriched diet for 28 weeks. *P<0.05 vs indicated group via Student unpaired t test.
male rabbits to examine the effects of natural androgens on lipids. One arm of the study involved bilateral castration of 20 male rabbits, whereas 20 were sham operated. Animals were fed an atherogenic diet for a 30-week period to induce aortic atherosclerosis. A doubling of aortic plaque formation was reported after castration in male rabbits compared with sham-operated controls. Similarly, Nathan et al7 assessed the effects of testosterone on early atherogenesis in orchidectomized LDL receptor knockout mice fed a cholesterol-enriched diet for a period of 8 weeks. In one arm of the study, the extent of lesion formation in the aortic region of male mice with testosterone levels in the subphysiological range (orchidectomized) was reported to be exacerbated compared with testes-intact animals. A number of clinical studies also have examined the association between serum levels of testosterone and coronary artery disease in men. These studies differ in their definition of atherosclerosis and in size but are consistent in their conclusion that endogenous serum levels of testosterone are not raised in men with coronary artery disease. In fact, the majority of these studies report that serum testosterone levels are reduced in men with coronary artery disease. Furthermore, low endogenous testosterone in men also is reported to be associated with high serum levels of HDL cholesterol.29–33 suggesting an association between hypotestosteronemia and a proatherogenic lipid profile, consistent with the observations of the present study. Testosterone replacement therapy results in a decrease in TC and HDLC levels of cholesterol in the Tfm mouse. Human cross-sectional studies have demonstrated a positive association between testosterone concentrations and HDLC in both healthy men36 and men with type 2 diabetes mellitus.37

In the present study, the degree of aortic lipid deposition was still significantly lower than that of placebo-treated Tfm mice with testosterone levels in the subphysiological range (orchidectomized) was reported to be exacerbated compared with testes-intact animals. However, a component of the effect of testosterone was not observed in Tfm mice receiving physiological testosterone replacement therapy alone, this effect was not observed in Tfm mice receiving physiological testosterone replacement in conjunction with either fulvestrant or anastrazole. Consequently, the beneficial action of testosterone in elevating HDLC levels would appear to occur by conversion to 17β-estradiol via aromatase and subsequent activation of ERα-dependent pathways. Indeed, previous studies have reported that physiological levels of 17β-estradiol may influence serum lipoprotein concentrations, although the results of these studies are conflicting.

The nongenomic mechanisms underlying the beneficial effect of testosterone on fatty streak formation are currently unknown. However, a number of nongenomic mechanisms of action of testosterone have recently been described, notably an ability to inhibit Ca1,1.2 L-type calcium channels in a manner similar to nifedipine. Indeed, nifedipine has been demonstrated to exert antiatherogenic effects in cholesterol-fed animal models without reducing dietary hypercholesterolemia. Nifedipine has been demonstrated to inhibit monocyte-macrophage-induced oxidation of LDL cholesterol and cholesterol accumulation, thereby reducing foam cell formation. Numerous studies report nongenomic effects of testosterone in macrophages similar to nifedipine (reviewed elsewhere). The extravasation of monocytes into the subendothelial cell layer is governed by several factors, including the expression of adhesion molecules and various cytokines. Testosterone has been demonstrated to decrease interleukin-6, interleukin-1β, and tumor necrosis factor-α production in monocyte-macrophages, as well as vascular cell adhesion molecule-1 expression and the nuclear translocation of nuclear factor-κB in human endothelial cells (reviewed by Jones et al). In addition, testosterone therapy has been reported to improve insulin sensitivity and to reduce elevated plasminogen activator inhibitor-1 levels in hypogonadal men (reviewed elsewhere). It is likely that 1 of these mechanisms underlie the observed beneficial effects of testosterone on fatty streak formation. Clearly, further study is warranted.

In summary, this study has demonstrated a beneficial action of testosterone on fatty streak formation. We have demonstrated that physiological concentrations of testosterone inhibit aortic fatty streak formation in cholesterol-fed mice, an action that is independent of the classic AR and
mediated in part by conversion to 17β-estradiol. In addition, physiological testosterone treatment increased HDLC, an effect consistent with conversion to 17β-estradiol and subsequent activation of genomic, ERα-dependent pathways. These data suggest that physiological testosterone replacement therapy may protect against atherogenesis and potentially confer cardiovascular benefits to men with hypotestosteronemia.

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Disclosures

None.

References


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**CLINICAL PERSPECTIVE**

Research indicates that low testosterone rather than testosterone per se is associated with coronary artery disease in men. Evidence also suggests that men with hypotestosteronemia with concomitant coronary artery disease may benefit from replacement therapy. The mechanisms by which testosterone produces these beneficial effects have yet to be elucidated. The testicular feminized (Tfm) mouse exhibit a nonfunctional androgen receptor (AR) and low circulating testosterone concentrations. We used the Tfm mouse to determine whether testosterone modulates atheroma formation via its classic signaling pathway involving the nuclear AR, conversion to 17β-estradiol, or an alternative signaling pathway. This study demonstrates that low endogenous testosterone, be it associated with a nonfunctional AR (in the Tfm mouse) or a fully functional AR (in the castrated XY male), is associated with fatty streak formation within the aortic root after feeding on cholesterol-enriched diet. Physiological testosterone replacement therapy was shown to prevent aortic fatty streak formation in the Tfm mouse and to raise high-density lipoprotein cholesterol. After testosterone replacement therapy in conjunction with either anastrozole or Faslodex, however, this protective effect was reduced although still significantly lower than that of placebo-treated Tfm mice. Improvement in high-density lipoprotein cholesterol also was completely abolished by cotreatment with these agents. These data provide evidence of a dual modulating effect of testosterone and 17β-estradiol in providing a beneficial action on fatty streak formation. Physiological testosterone replacement inhibited aortic fatty streak formation in cholesterol-fed mice, an action that was independent of the classic AR. Additionally, physiological testosterone replacement increased high-density lipoprotein cholesterol, an effect consistent with conversion to 17β-estradiol and subsequent activation of genomic, estrogen receptor α–dependent pathways.
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