

Minoxidil Sulfate Is the Active Metabolite that Stimulates Hair Follicles

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An important step in understanding minoxidil's mechanism of action on hair follicles was to determine the drug's active form. We used organ-cultured vibrissa follicles to test whether it is minoxidil or its sulfated metabolite, minoxidil sulfate, that stimulates hair growth. Follicles from neonatal mice were cultured with or without drugs and effects were assessed by measuring incorporation of radiolabeled cysteine in hair shafts of the treated follicles. Assays of minoxidil sulfotransferase activity indicated that vibrissae follicles metabolize minoxidil to minoxidil sulfate. Dose-response studies showed that minoxidil sulfate is 14 times more potent than minoxidil in stimulating cysteine incorporation in cultured follicles. Three drugs that block production of intra-follicular minoxidil sulfate were tested for their effects on drug-induced hair growth. Diethylcarbamazine proved to be

a noncompetitive inhibitor of sulfotransferase and prevented hair growth stimulation by minoxidil but not by minoxidil sulfate. Inhibiting the formation of intracellular PAPS with chlorate also blocked the action of minoxidil but not of minoxidil sulfate. Acetaminophen, a potent sulfate scavenger blocked cysteine incorporation by minoxidil. It also blocked follicular stimulation by minoxidil sulfate apparently by directly removing the sulfate from the drug. Experiments with U-51,607, a potent minoxidil analog that also forms a sulfated metabolite, showed that its activity was inhibited by both chlorate and diethylcarbamazine. These studies show that sulfation is a critical step for hair-growth effects of minoxidil and that it is the sulfated metabolite that directly affects hair follicles. *J Invest Dermatol* 95:553–557, 1990

Minoxidil is a potent vasodilator that also induces hypertrichosis of facial and body hair [1–4]. Studies from this laboratory show that minoxidil directly affects cultured vibrissa follicles by enhancing both proliferation and differentiation of the epithelium that comprises the hair shaft [5]. Additional studies indicate that minoxidil induces a rapid expression of genes for both c-fos and a hair protein in vitro [6]. Although the exact mechanisms of minoxidil's action on hair follicles are unknown, this work suggests that minoxidil's action on hair follicles is direct and that vasodilation or enhanced blood flow are not causal mechanisms. Understanding how minoxidil works will provide useful insights into the regulation of hair growth.

Characterization of minoxidil's vasodilator activity indicates that it is the sulfated metabolite, minoxidil sulfate, that affects vascular smooth muscle (see Fig 1 for structures). Both in vivo and in vitro studies show that minoxidil sulfate and not the parent drug causes a rapid relaxation of vascular smooth muscle [7–10]. The sulfation of minoxidil is catalyzed by sulfotransferase. This activity has been demonstrated in liver, platelet, skin, and coat follicles but has not been extensively characterized [11–13]. Minoxidil sulfate is inherently unstable in an aqueous solution being hydrolyzed to the parent drug [14].

This report describes our efforts to determine if it is minoxidil or

minoxidil sulfate that stimulates cultured vibrissa follicles. To inhibit the formation of minoxidil sulfate in follicles, we used three drugs. Two of these, chlorate and diethylcarbamazine, interfere with the enzymes involved in intercellular metabolism of sulfate (Fig 2). Chlorate inhibits ATP-sulfurylase, the first enzyme in the synthesis of PAPS [15]. Diethylcarbamazine inhibits the sulfation of proteoglycans from PAPS [16] and, as we demonstrate in this report, is a noncompetitive inhibitor of sulfotransferase. Acetaminophen is a competitive substrate that blocks sulfation of minoxidil by depleting intracellular stores of the activated sulfate donor PAPS [17].

To test the hypothesis that minoxidil sulfate is the active drug for hair growth, we first assayed follicles and demonstrated that these organs have sulfotransferase activity. Next, we compared the potency of minoxidil and minoxidil sulfate and showed that the sulfate is more active than minoxidil in stimulating the cultured follicles. Then, we used the three inhibitors to demonstrate that drugs that block the production of minoxidil sulfate prevent minoxidil's action in follicles, but that these drugs fail to inhibit the effects of minoxidil sulfate. Additional experiments with an active minoxidil analog, U-51,607, showed that this analog also must be sulfated to stimulate follicles in culture.

MATERIAL AND METHODS

Assay of Sulfotransferase Activity Measurement of minoxidil sulfotransferase using a human-platelet homogenate as the enzyme source has been described previously [12]. Briefly, this assay measures the amount of ³⁵S-minoxidil sulfate formed by the enzyme-catalyzed transfer of ³⁵S-labeled PAPS (New England Nuclear, Boston, MA) to the N-oxide of minoxidil. Inhibition of sulfotransferase by diethylcarbamazine was tested by adding different amounts of the drug to the incubation mixture.

The minoxidil analog, U-51,607, was shown to form a sulfated metabolite, using the same methods for measuring sulfotransferase

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Abbreviation:

PAPS: 3'-phosphoadenosine 5'-phosphosulfate

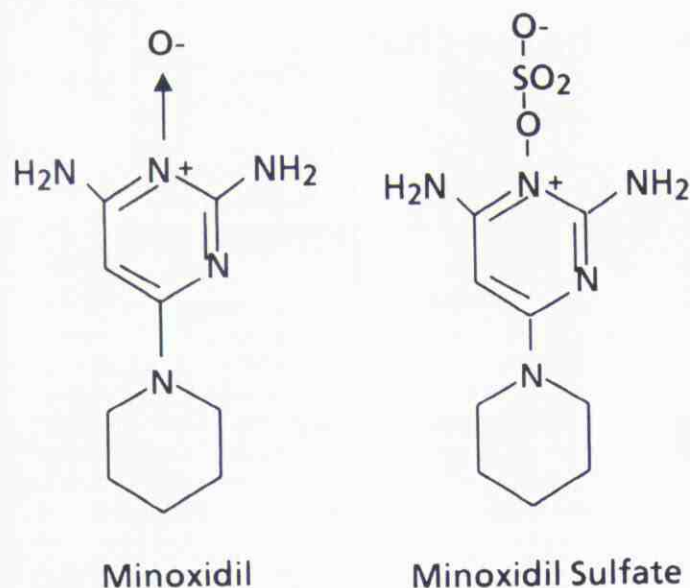


Figure 1. Structures of minoxidil and minoxidil sulfate.

described above [12]. Unlabeled drug was incubated with human-platelet homogenate and transfer of ^{35}S from the ^{35}S -labeled PAPS indicated that the analog formed a sulfated metabolite.

Minoxidil sulfotransferase activity in mouse vibrissae was determined after homogenizing 40 freshly dissected follicles in 3 ml of cold 50-mM KPO_4 buffer, pH 7.3, with a Polytron (Brinkman Instruments, Westbury, NY). The resulting homogenate was centrifuged at $100,000 \times g$ for 45 min at 4°C . Enzyme activity in duplicate 100- μl aliquots was determined using the assay described above.

Measurement of Follicle Stimulation Drug effects on hair follicles were assayed by measuring incorporation of ^{35}S -cysteine into the newly forming hair shafts of cultured follicles, using our previous published methods [5,18]. Intact vibrissa follicles were harvested from F_1 hybrid mice ($\text{C57BL6} \times \text{C3H}$) at 3–4 d of age. Groups of 5–7 follicles were placed in individual wells of 24-well tissue culture plates (Corning, Corning, NY) and incubated with drug for 72 h at 37°C with 10% CO_2 in specially formulated Dulbecco's Modified Eagle's Medium without cysteine (GIBCO, Grand Island, NY). ITS, a medium supplement containing insulin, transferin, and selenium, was added to the medium (Collaborative Research, Bedford, MA). At 24 h, 10 $\mu\text{Ci}/\text{ml}$ of ^{35}S -cysteine (Amersham, Arlington Heights, IL) was added to each well and at 72 h the incorporation of label into individual hair shafts was measured using a scintillation counter. Serum-free medium was used because preliminary experiments showed that fetal bovine serum contains sulfotransferase activity.

Potency comparisons between minoxidil and minoxidil sulfate or the minoxidil analog, U-51,607, were done in single assays containing control medium and varying dosages of each drug. The influence of the drugs affecting sulfate production (chlorate, diethylcarbamazine, and acetaminophen) on the activity of three minoxidils (minoxidil, minoxidil sulfate, and U-51,607) was tested in a second set of experiments. The effects of each inhibitor were measured alone and in combination with each of the three minoxidils. To maximize the effects of the sulfotransferase inhibitors, these drugs were added to the follicles 1–3 h before the addition of the minoxidils.

Minoxidil and U-51,607 [pyrimidine 2,4 diamino-6-hexa-

mino-, 3-oxide] (The Upjohn Company, Kalamazoo, MI) were dissolved in the culture medium at concentrations between 0.01 and 1.0 mM. Sodium chlorate (Baker Chemical Company, Phillipsburg, NJ), diethylcarbamazine, and acetaminophen (Sigma Chemical Company, Milwaukee, WI) were also dissolved in media at 1-mM concentrations. Minoxidil sulfate (Upjohn) was dissolved in acetone at 100 times excess and added directly to the media in the wells; the final concentrations ranged between 0.0007 and 0.07 mM. The media in these minoxidil sulfate groups contained 2% acetone. Acetone was also added to the media containing the other drugs and to the control media. Previous experiments showed that this concentration of acetone has no effect on cultured follicles.

Statistics All experiments were analyzed using analysis of variance. The dose-response comparison between minoxidil and minoxidil sulfate was done with a one-way analysis followed by least-squares means tests or t tests. The effects of diethylcarbamazine, chlorate, and acetaminophen were tested in individual experiments and analyzed using a two-way analysis with replication. A significance level of $p < 0.05$ was used throughout these experiments.

RESULTS

Inhibition of Sulfotransferase by Diethylcarbamazine

Diethylcarbamazine inhibits the activity of platelet sulfotransferase when tested at doses of 5–15 mM. Presentation of these data in the form of a Lineweaver-Burk plot shows that the action of diethylcarbamazine is that of a noncompetitive inhibitor (Fig 3).

Sulfotransferase Activity in Follicles Freshly dissected vibrissae clearly have the capacity to convert minoxidil to minoxidil sulfate. Sulfotransferase activity measured in five different groups of mouse vibrissae formed 36 ± 2 (mean $\pm$ SEM) fmoles of minoxidil sulfate per follicle.

To localize the enzyme activity, three groups of follicles were dissected into pieces comprised of shaft (containing the hair shaft plus cortex, medulla, inner root sheath, and some outer root sheath) or sheath (containing the dermal papilla, remainder of the outer root sheath, plus the cavernous sinus and the associated connective tissue). The process of dissection causes a loss of tissue and results in a loss of 46% of the enzyme. After dissection, the sheath portion contained 21.6 ± 2.4 and the shaft only 2.1 ± 0.8 fmoles of activity.

Formation of the Sulfated Metabolite of U-51,607 Incubation of the minoxidil analog, U-51,607 with labeled PAPS and platelet homogenate showed that this also is a substrate for sulfotransferase. The amount of labeled sulfate transferred to the analog was 63,039 cpm at 1.5 mM substrate concentration; in comparison, the amount transferred to 1.5 mM minoxidil was 27,368 cpm, whereas the blank in the experiment was 769 cpm. These results are

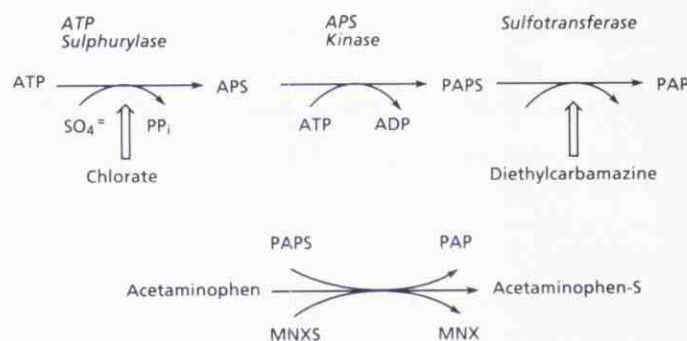


Figure 2. Steps at which drugs interfere with sulfation of minoxidil. Inorganic phosphate (PPi), adenosine 5'-phosphosulfate (APS), 3'-phosphoadenosine 5'-phosphosulfate (PAPS), 3'-phosphoadenosine 5'-phosphate (PAP), minoxidil (MNX), minoxidil sulfate (MNXS).

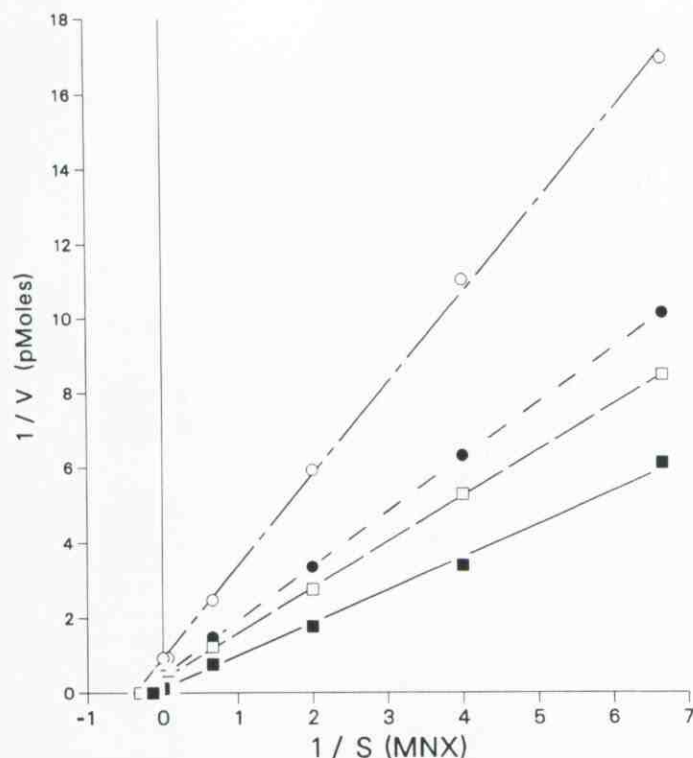


Figure 3. Lineweaver-Burk plot showing the interaction of diethylcarbamazine on sulfotransferase activity as measured by production of minoxidil sulfate from minoxidil (MNX). Diethylcarbamazine concentrations are 0 mM (■), 5.0 mM (□), 7.4 mM (●), and 14.6 mM (○).

the mean of duplicate samples and are typical of several replicates of this experiment.

Dose Comparison Between Minoxidil and Minoxidil Sulfate Minoxidil sulfate is more potent than minoxidil in stimulating cysteine incorporation in cultured follicles (Fig 4). Minoxidil sulfate at 0.007 mM stimulates cysteine uptake to the same degree as minoxidil at 0.1 mM. Similarly, the follicles were stimulated equally by minoxidil sulfate at 0.07 mM and minoxidil at 1.0 mM. The potency difference between the parent drug and the sulfate is approximately 14 times. Several replicates of the experiment verified this difference in minimal effective dose.

Separate experiments showed that the relative potencies between the minoxidil analog U-51,607 and minoxidil was about 10 times. Mean cysteine uptake ($\pm$ SEM) in groups of six to eight follicles was 897 ± 61 cpm for 0.5 mM minoxidil compared to 841 ± 123 for 0.05 mM U-51,607. Both drugs increased uptake as compared to the control group, 462 ± 68 cpm. These potency comparisons were also replicated in other experiments.

Effect of Sulfate Inhibitors Diethylcarbamazine blocked the stimulatory effects of minoxidil in cultured follicles (Fig 5). However, this non-competitive inhibitor of sulfotransferase did not block the stimulatory effects of minoxidil sulfate. Uptake of radio-labeled cysteine was significantly stimulated by both minoxidil and minoxidil sulfate alone, as compared to control medium without drug. Diethylcarbamazine alone did not influence uptake of labeled cysteine.

Addition of chlorate inhibited the minoxidil-induced stimulation of the cultured follicles but did not affect the stimulatory activity of the sulfate (Fig 6). Both minoxidil and the sulfate were active when given without this PAPS inhibitor. Chlorate by itself had no effect on cysteine uptake compared to the control medium containing no drug.

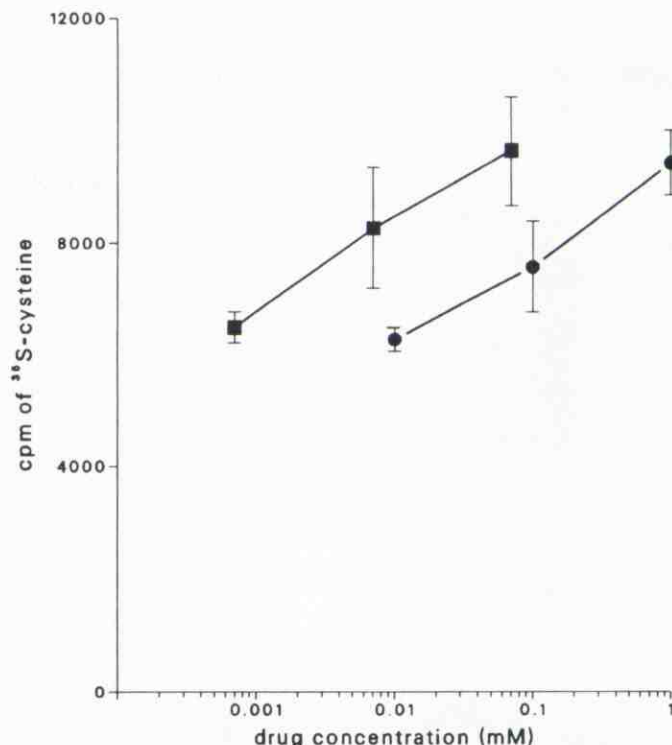


Figure 4. Potency comparison between minoxidil (●) and minoxidil sulfate (■) as measured by incorporation of ^{35}S -cysteine. The two higher doses of both drugs were significantly greater than the control group without drug treatment (5847 ± 310 , mean $\pm$ SEM). Each group contained five follicles, counts are per follicle; bars, mean $\pm$ SEM.

Acetaminophen blocked the stimulation of cultured follicles by both minoxidil and minoxidil sulfate (Fig 7). Control follicles cultured with either minoxidil or the sulfate without acetaminophen showed significant stimulation. Acetaminophen alone did not reduce cysteine incorporation compared to control follicles without any drugs.

Similar results were obtained when testing the minoxidil analog, U-51,607, with the drugs that interfere with sulfation. Both diethylcarbamazine and chlorate blocked the activity of U-51,607.

DISCUSSION

These experiments provide the first evidence that minoxidil's effects on hair follicles are due to the sulfated metabolite, minoxidil sulfate. This conclusion rests on the data showing that the sulfotransferase enzyme is present in follicles, that minoxidil sulfate is more potent than minoxidil in stimulating cultured follicles, and that drugs that block formation of minoxidil sulfate block the activity of the parent drug but not of the sulfated metabolite. Results with the active minoxidil analog suggest that the requirement for sulfation is generalized among the minoxidil family. Experiments to determine if the requirement for sulfation extends to non-minoxidil-like drugs that cause hypertrichosis are in progress. Clearly, sulfation of minoxidil is a critical step for its effects on hair growth.

Our finding that minoxidil sulfate is the active metabolite in hair follicles was not unexpected because previous studies showed similar results on vascular smooth muscle. Perfusion of an isolated vascular bed with minoxidil had no effect, whereas minoxidil sulfate relaxed the blood vessels [9]. This experiment was successful because the vasculature has little sulfotransferase activity and effects on vascular smooth muscle are rapid. Other results show that minoxidil-induced gene expression occurs within 2 h in cultured vi-

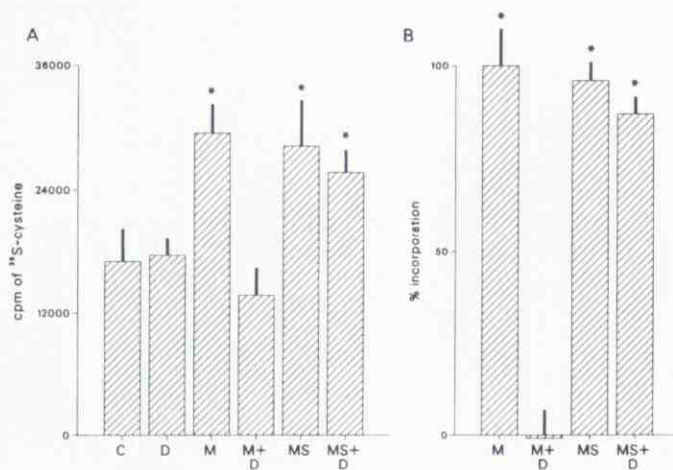


Figure 5. Influence of diethylcarbamazine (D) on follicles treated with minoxidil (M) or minoxidil sulfate (MS). The control (C) group received no drug treatment. A) This representative experiment shows drug effects on ³⁵S-cysteine incorporation. Each group contains five follicles. B) A summary of three replicate experiments with stimulation calculated as percent of minoxidil. Bars, mean $\pm$ SEM; asterisks, significant difference from the control group, $p < 0.05$.

brissa [6]. Some similarity in mechanism of action must be present in both systems beyond the requirement for the sulfation of minoxidil.

Our data showing minoxidil sulfotransferase activity in mouse vibrissa follicles confirms a recent report showing sulfotransferase activity in isolated coat follicles and cultured keratinocytes from rats [13]. We found the majority of the activity in the sheath part of the vibrissa. Other efforts are underway to obtain histochemical evidence for localizing minoxidil sulfotransferase in hair follicles.

Several types of agents that interfere with the *in vivo* transfer of sulfate have been identified. Various phenols, including acetaminophen and chlorophen, have been used as competitive substrates and are effective in reducing the availability of the active sulfate through PAPS [17]. Other drugs, including 2,6-dichloro-4-nitrophenol and pentachlorophenol are effective competitive inhibitors of sulfotransferase and are not sulfated *in vivo*. We chose not to use the latter set of drugs due to their reported toxicity.

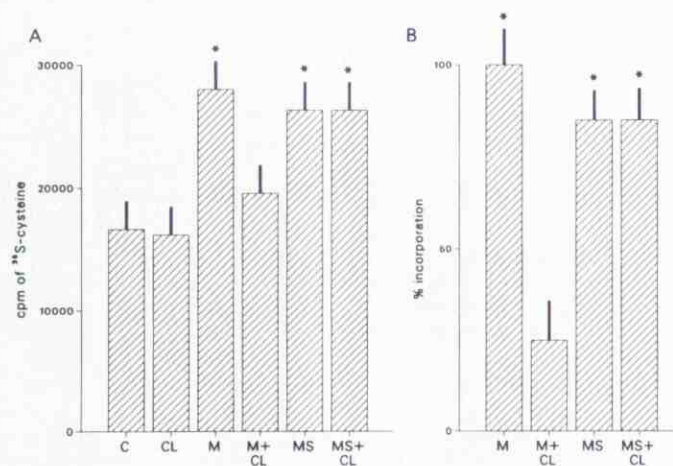


Figure 6. Effects of chlorate (CL) on follicles treated with minoxidil (M) or minoxidil sulfate (MS). The control (C) group received no drug treatment. A) This representative experiment shows drug effects on ³⁵S-cysteine incorporation. Each group contains five follicles. B) A summary of two replicate experiments with stimulation calculated as percent of minoxidil. Bars, mean $\pm$ SEM; asterisks, significant difference from the control group, $p < 0.05$.

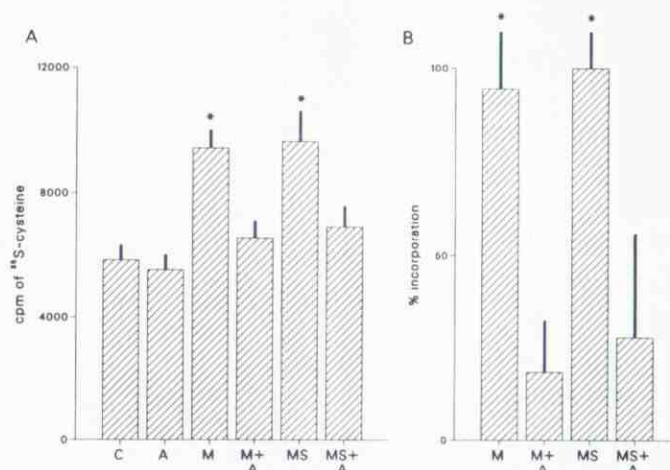


Figure 7. Acetaminophen's (A) effects on follicles treated with minoxidil (M) or minoxidil sulfate (MS). The control (C) group received no drug treatment. A) This representative experiment shows drug effects on ³⁵S-cysteine incorporation. Each group contains five follicles. B) A summary of duplicate experiments with stimulation calculated as percent of minoxidil. Bars, mean $\pm$ SEM; asterisks, significant difference from the control group, $p < 0.05$.

The data obtained using the sulfotransferase inhibitors supports the hypothesis that minoxidil sulfate is active in hair follicles. Both chlorate and diethylcarbamazine block the transfer of sulfate through the PAPS system by different methods. Chlorate inhibits the formation of PAPS, whereas diethylcarbamazine is a noncompetitive inhibitor of the sulfotransferase enzyme [15,16]. Both blocked follicle stimulation by minoxidil but not by minoxidil sulfate.

Our data with acetaminophen also support the hypothesis that minoxidil sulfate is the active agent, even though it blocked the action of the sulfate in our studies. Because acetaminophen is a sulfate scavenger, it accumulates sulfate directly from PAPS but would also be expected to remove sulfate directly from minoxidil sulfate [17]. Thus, acetaminophen eliminates both the minoxidil sulfate generated within the hair follicle and the exogenously administered drug.

Knowing that minoxidil sulfate is the active metabolite in hair follicles has several implications. First, it provides a specific chemical tool for examining the biochemical mechanisms that regulate hair follicles. We can now design further studies to understand how this sulfated metabolite of minoxidil works on the follicular cells. Second, because the sulfate is active, variations in the clinical response to minoxidil between individuals may be due to differences in endogenous production of minoxidil sulfate. Differing amounts of sulfotransferase or differences in isoenzymes may explain why topical minoxidil works better in some patients than in others. Verification of this hypothesis is underway. Finally, our data suggest that other drugs that can be metabolized to an active sulfate might be expected to directly affect hair follicles.

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REFERENCES

1. Earhart RN, Ball J, Nuss DD, Aeling JL: Minoxidil induced hypertrichosis: treatment with calcium thioglycolate depilatory. *South Med J* 70:442-443, 1977

2. Dargie HJ, Dollery CT, Daniel J: Minoxidil in resistant hypertension. *Lancet* II:515–518, 1977
3. Devine BL, Fife R, Trust PM: Minoxidil for severe hypertension after failure of other hypertensive drugs. *Br Med J* 2:667–669, 1977
4. Headington JT, Novak E: Clinical and histological studies of male pattern baldness treated with topical minoxidil. *Curr Ther Res Clin Exp* 36:1098–1106, 1984
5. Buhl AE, Waldon DJ, Kawabe TT, Holland JM: Minoxidil stimulates mouse vibrissae follicles in organ culture. *J Invest Dermatol* 92:315–320, 1989
6. Rea T, Buhl AE, McNab A, Kawabe TT, Waldon DJ, Vogeli G, Groppi V: Regulation of gene expression by minoxidil. *J Invest Dermatol* 92:504, 1989
7. DuCharme DW, Freyburger WA, Graham BE, Carlson RG: Pharmacological properties of minoxidil: a new hypotensive agent. *J Pharmacol Exp Ther* 184:662–670, 1973
8. Baker CA, Hansen JP, Williams MV, Robinson FG, Rogers JE, Zandt RA, Wendling MG, Johnson GA: Relationship between the physiologic activity of minoxidil and tissues levels of minoxidil and minoxidil sulfate. *Fed Proc* 43:556, 1984
9. McCall JM, Aiken JW, Chidester CG, DuCharme DW, Wendling MG: Pyrimidine and triazine 3-oxide sulfates: a new family of vasodilators. *J Med Chem* 26:1791–1793, 1983
10. Meisneri KD, Cipkus LA, Taylor CJ: Mechanism of action of minoxidil sulfate-induced vasodilation: a role for increased K⁺ permeability. *J Pharmacol Exp Ther* 245:751–760, 1988
11. Roy AB: Sulfotransferases. In: Mulder GJ, (ed.). *Sulfation of drugs and related compounds*. CRC Press, Boca Raton, FL, 1981, pp 83–130
12. Johnson GA, Baker CA: Sulfation of minoxidil by human platelet sulfotransferase. *Clin Chim Acta* 169:217–228, 1987
13. Hamamoto T, Mori Y: Sulfation of minoxidil in keratinocytes and hair follicles. *Res Comm Chem Path Pharm* 66:33–44, 1989
14. Johnson GA, Barsuhn KJ, McCall JM: Sulfation of minoxidil by liver sulfotransferase. *Biochem Pharmacol* 31:2949–2954, 1982
15. Baeuerle PA, Huttner WB: Chlorate—a potent inhibitor of protein sulfation in intact cells. *Biochem Biophys Res Comm* 141:870–877, 1986
16. Stevens RL, Parsons WG, Austen KF, Hein A, Caulfield JP: Novel inhibition of proteoglycan synthesis and exocytosis by diethylcarbamazine in the swarm rat chondrocyte. *J Biol Chem* 260:5777–5786, 1985
17. Reiter C, Weinshilboum RM: Acetaminophen and phenol: substrates for both a thermostable and a thermolabile form of human platelet phenol sulfotransferase. *J Pharmacol Exp Therap* 221:43–51, 1982
18. Buhl AE, Waldon DJ, Kawabe TT: Hair growth in cultured mouse vibrissae follicles. *In Vitro* 24:28A, 1988