Biochemical Pharmacology, Vol. 45, No. 2, pp. 271–279, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

COMMENTARY

ENZYMATIC AND NON-ENZYMATIC SULFATION MECHANISMS IN THE BIOLOGICAL ACTIONS OF MINOXIDIL

KAUSHIK D. MEISHERI,*† GARLAND A. JOHNSON‡ and LYNN PUDDINGTON§‖
*Cardiovascular Diseases, ‡Dermatology, and §Cell Biology Research, Upjohn Laboratories, The Upjohn Company, Kalamazoo, MI 49001, U.S.A.

An analysis of the scientific literature regarding minoxidil suggests that serendipitous observations coupled with experimental pursuit of these observations by a small number of investigators have played important roles during the discovery and development of minoxidil as an antihypertensive as well as a hair growth promoting agent. This is also true for the work done subsequently towards defining the cellular mechanism of action of minoxidil. This review will describe some of the salient features of the discovery of minoxidil as a unique drug entity, and will illustrate how this compound has become a valuable tool for exposing some unique functional capacities of cells. These include identification of a sulfotransferase enzyme responsible for bioactivation of minoxidil, identification of a K^+ channel opening mechanism for vasodilation, and identification of protein substrates for post-translational non-enzymatic sulfate addition.

The origins of the discovery of minoxidil can be traced back more than 30 years to the identification of another antihypertensive agent at The Upjohn Company. The original compound diallylmelamine (U-7720) was a lead gastric antisecretory agent. Its cardiovascular evaluation revealed pronounced hypotension in unanesthetized dogs. Subsequent work showed that diallylmelamine is a direct acting peripheral vasodilator that has a delayed onset of action when given orally to rats and dogs [1]. Surprisingly, this agent was inactive as a hypotensive agent when given orally to humans. This sparked intense investigation of the metabolic fate of this compound. In an elegant series of experiments performed in rats, dogs and humans, Zins and co-workers [2, 3] demonstrated that diallylmelamine is converted to the ring *N*-oxide *in vivo*, and this metabolite is responsible for the hypotensive activity. They further demonstrated that humans are deficient in the capacity to make this metabolic conversion which accounted for the inactivity of U-7720 in man [3]. Based on this, subsequent chemical developments at The Upjohn Company led to the synthesis of a second generation of compounds from which minoxidil (U-10858) was selected for development as an antihypertensive agent in the early 1970s. During this time period, one of the clinical treatments for severe and malignant hypertension in patients who did not respond to antihypertensive medications was nephrectomy followed by chronic renal dialysis. Minoxidil proved to be quite an effective agent in the drug therapy of these patients with malignant hypertension, and continues to be the drug of choice for the treatment of hypertension that is not controlled satisfactorily by other antihypertensive drugs.

Metabolic activation of minoxidil: The role of enzymatic sulfation

It will be of interest to the reader to review here the experimental evolution of the concept that metabolic activation of minoxidil via enzymatic sulfation is necessary for its hypotensive activity *in vivo*. Two characteristics of minoxidil stood out during the early studies of its effects in animals. One was the 15- to 60-min delay in onset of hypotensive activity, regardless of the route of administration [4]. This observation was further accentuated by the fact that the maximum decrease in blood pressure occurred 1–2 hr after the maximum levels of drug were detected in the plasma [4, 5]. Second was the prolonged hypotensive effect lasting 48–72 hr following administration. In fact, the hypotensive response continued long after nearly all of the drug (>95% of the labeled drug) had been excreted from the body [6]. These observations inferred that a metabolite of minoxidil that caused an irreversible change in vascular smooth muscle was responsible for its pharmacological activity. Subsequently very thorough metabolic studies in four species, i.e. rat, dog, monkey, and human, resulted in the isolation and identification of several metabolites [7–9]. Synthesis of the identified metabolites and their study in various hypertensive animal models showed that none of these molecules accounted for the pharmacological activity of minoxidil.

Nevertheless animal studies continued to suggest that metabolic activation of minoxidil was required for biological activity.¶ For example, the hypotensive activity of minoxidil in conscious dogs was altered markedly by pretreatment of the animals with the mixed-function oxidase inhibitor SKF 525-A. Further, addition of minoxidil to a closed circuit,

† Corresponding author: Dr. Kaushik D. Meisheri, Cardiovascular Diseases Research, 7243-209-315, Upjohn Laboratories, Kalamazoo, MI 49001. Tel. (616) 385-7949; FAX (616) 385-5262.
‖ Current address: Department of Medicine, MC 1310, University of Connecticut Health Center, Farmington, CT 06030.
¶ DuCharme DW and Wendling MG, personal communication. Cited with permission.

blood perfused dog gracilis muscle preparation did not alter the vascular resistance; however, if the blood which contained minoxidil was allowed to circulate through the animal and then back into the perfusion circuit, vasodilation ensued. Description of another vasodilator, Ro 12-4713 [10], which structurally resembled minoxidil, also spurred further interest. These observations prompted a study in which rats were cannulated for bile collection and dosed intraarterially with [^{14}C]minoxidil. Subsequently, the radioactive bile samples were collected and peaks were isolated and assayed for vasorelaxing activity.* One radiolabeled fraction, always obtained late in the afternoon of the day of the cannulation experiment, frequently produced an immediate decrease in perfusion pressure in the isolated rat hind limb preparation without affecting perfusion pressure in the contralateral limb. Studies performed the next morning with the same fraction frequently failed to reproduce the activity, suggesting the evanescent nature of the "relaxing factor". Further examination of bile fractions showed that the vasorelaxing activity was retained on an anion exchange column, although not very efficiently. This hint of an anionic nature of the metabolite and work on sulfate conjugates of catecholamines [11] led to the speculation that a sulfate conjugate of minoxidil was the active molecule. The fact that injecting rats with large doses of a sulfate scavenger, acetaminophen, essentially blocked the hypotensive activity of minoxidil supported this hypothesis. Subsequently, a sulfotransferase enzyme activity from rat liver which is able to catalyze the formation of the *N-O* sulfate of minoxidil and several other pyrimidine-, pyridine, triazine-, and imidazole-*N*-oxides *in vitro* was isolated and characterized [12]. The intriguing and often frustrating chase for the active metabolite finally appeared to come to fruition when minoxidil sulfate was identified as a naturally occurring metabolite in rats treated with minoxidil [13]. The ultimate confirmation came from the chemical synthesis of minoxidil sulfate which was shown to be more potent and to have a faster onset of vasodilatory action than minoxidil [14]. X-ray crystallography verified that a sulfate moiety is conjugated to minoxidil on the *N*-oxide (Fig. 1). It was also shown that in an *in vitro* preparation of rabbit mesenteric artery smooth muscle, minoxidil sulfate produces relaxation whereas minoxidil is inactive [14]. Thus, the identification of minoxidil sulfate satisfied almost all of the unanswered questions regarding the *in vivo* pharmacological and pharmacokinetics profile of minoxidil. It is now known that certain physico-chemical peculiarities of minoxidil sulfate prevented its detection for a number of years. For example, minoxidil sulfate shows marked instability in alcohol-containing solvents and also in aqueous solutions. The half-life of minoxidil's sulfate is 6 hr in aqueous solution and much less in alcohol-containing solvents. Also, in contrast to most sulfate esters, minoxidil sulfate is more hydrophobic

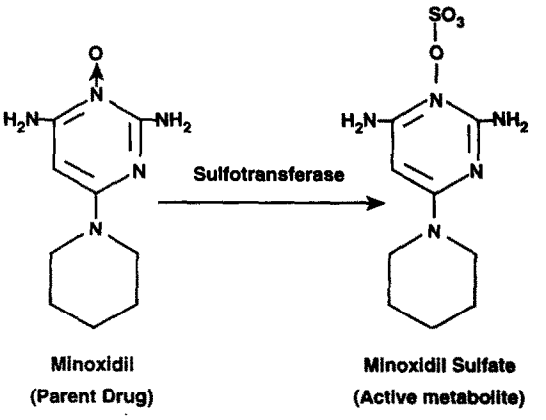

Fig. 1. Enzymatic bioactivation of minoxidil.

than minoxidil due to the formation of an inner salt, and minoxidil sulfate is not readily detectable in the urine or bile.

It should be noted that the enzymatic sulfation as a mechanism of bioactivation of minoxidil is unique. To date, this remains as the only example for the bioactivation of a therapeutic drug via such a transformation. In contrast, sulfation of several compounds has been implicated in their conversion to carcinogens, eliminating them from consideration as therapeutic agents. In most instances, sulfate conjugation is associated with a decrease in the biological activity and an increase in the excretion [15].

Requirement of enzymatic sulfation for the hair growth effect of minoxidil

Minoxidil was originally developed and approved for use in the treatment of hypertension [16]. The observation that minoxidil caused hair growth stimulation in patients being treated for hypertension led to the development of a topical minoxidil solution for the treatment of male pattern baldness. Minoxidil remains the only approved clinical treatment for baldness in men and women. An understanding of the metabolic activation of minoxidil for its hypotensive effect raised the question of whether the same bioactivation was required for this other important biological effect of minoxidil. Efforts to address this question experimentally became possible with the development of an organ culture system whereby mouse vibrissae follicles cultured *in vitro* represent biological activities associated with hair growth *in vivo* [17†] Using ^{35}S-labeled cysteine incorporation as an indicator of biogenesis of hair keratins, it has been demonstrated that both minoxidil and minoxidil sulfate stimulate hair growth [18]. In this *in vitro* culture system, minoxidil sulfate was found to be more potent than minoxidil. The inhibitors of sulfotransferase (diethylcarbamazine, chlorate) or the sulfate scavenger (acetaminophen) inhibited the actions of minoxidil but not of minoxidil sulfate, suggesting that conversion of minoxidil to minoxidil sulfate is a critical step in the action of

* DuCharme DW and McCandlis MR, personal communication. Cited with permission.

† Puddington L, Clawitter JL and Buhl AE, manuscript in preparation.

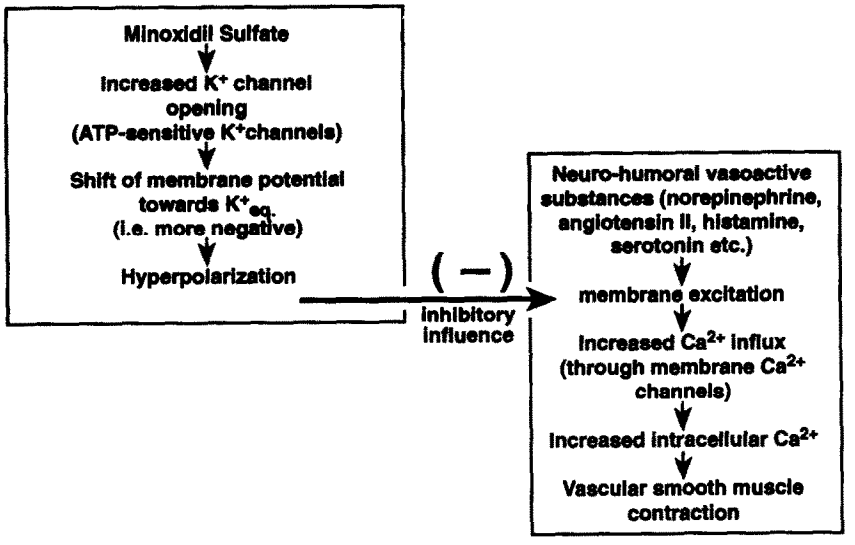

Fig. 2. Schematic depiction of the mechanism of action of minoxidil sulfate.

minoxidil on the hair follicle. Localization of the sulfotransferase enzyme responsible for bioactivation of minoxidil in the outer root sheath of vibrissa follicles [19] is consistent with the capacity of minoxidil to stimulate hair growth. These observations raise an interesting question, i.e. whether the differential degree of hair growth stimulating effects of topical minoxidil in humans is related to the differential degree of metabolic activation of minoxidil through this enzymatic sulfation pathway.

Minoxidil sulfotransferase

As indicated above, Johnson *et al.* [12] provided the first demonstration that minoxidil and several other analogues are sulfated by an enzyme in rat liver homogenates which catalyzes sulfation of *N*-oxide moieties. The resulting *N,O*-sulfate is less polar, i.e. more lipophilic, than the *N*-oxide substrate. Minoxidil sulfotransferase activity has since been demonstrated in human platelets [20], liver [21], scalp skin [22], hair follicles [22, 23], and epidermal keratinocytes [23, 24] as well as in mouse vibrissa follicles [19]. Purification and characterization of the minoxidil sulfotransferase activity present in human and rat liver have shown it to be a phenol sulfotransferase [21, 25].

Reiter *et al.* [26] described two phenol sulfotransferases in rat liver, one of which was thermal labile and responsible for the sulfation of monoamines, i.e. the "M-form" of phenol sulfotransferase (M-PST), and a second thermal stable form, the "P-form" (P-PST), responsible for sulfating phenolic substrates, i.e. *p*-nitrophenol. By affinity chromatography, Hirshey and Falany [25] have separated the minoxidil sulfotransferase activity of rat liver from a number of proteins (P-PST) that sulfate *p*-nitrophenol but not minoxidil. This activity migrates as a single band on sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE) and effects the sulfation of minoxidil and *p*-nitrophenol. They found minoxidil sulfotransferase activity in rat liver, but *not* in kidney, intestine, or brain, tissues containing easily demonstrable P-PST activity. In their attempts to isolate the minoxidil sulfotransferase activity from human liver, Falany and Kerl [21] were able to resolve, again by affinity column chromatography, the P-PST and M-PST activities. In their isolation procedures, minoxidil sulfotransferase co-purifies with thermal stable P-PST activity. In contrast, the minoxidil sulfotransferase activity in human platelets is predominantly thermal labile [20]. Taken together, the available data suggest that the minoxidil sulfotransferase activity in rat liver represents a "unique form of sulfotransferase" which has different characteristics when compared to the known isolated P-PSTs [25]. The molecular identification of this enzyme, which sulfates minoxidil, causing its bioactivation and increasing its lipophilicity, remains to be elucidated.

Minoxidil sulfate: Cellular mechanism of action

Although *in vivo* studies demonstrated minoxidil to be a vasodilator antihypertensive agent, the mechanism by which it produced this effect was unknown. The identification and chemical synthesis of the active metabolite, minoxidil sulfate, provided the opportunity to define mechanisms by which minoxidil sulfate relaxes blood vessels. These investigations, again, led to some unique observations. It was shown that the primary action of minoxidil sulfate on vascular smooth muscle is to open plasmalemmal potassium channels [27*]. As

* Meisheri KD, Taylor CJ and Cipkus LA, The mechanisms of vascular smooth muscle relaxing effects of minoxidil sulfate. In: *Smooth Muscle Function Symposium Proceedings: Official Satellite Symposium of the XXX International Physiology Congress, Banff, Canada, 1986*, p. 114.

illustrated in Fig. 2, opening of the K^+ channels in smooth muscle leads to hyperpolarization of the membrane which interferes with the ability of vasoconstrictors to increase intracellular Ca^{2+} and cause contraction. Thus, the end result of vascular K^+ channel opening is smooth muscle relaxation. When the description of this mechanism became known [27*], it demonstrated that minoxidil (sulfate) was the *first* clinically useful vasodilator that elicited its therapeutic effects via the K^+ channel opening mechanism. Since 1986, several other chemically distinct structures known to be vasodilators have been found to work through the same K^+ channel opening mechanism. Therefore, this new class of direct acting vasodilators is known as "Potassium Channel Openers", and includes the parent compounds and analogues of minoxidil sulfate, as well as pinacidil, diazoxide, and cromakalim [28]. As originally suggested by Winquist *et al.* [29], these agents have been found to specifically open the ATP-sensitive class of K^+ channels in vascular smooth muscle cells [30–32].

Interestingly, the same cellular mechanism of K^+ channel opening has been implicated in the action of minoxidil in stimulating hair growth [33]. Recently, it has been shown that like minoxidil, other K^+ channel opener vasodilators stimulate hair growth *in vitro* and *in vivo* [34]. It remains to be established whether the *in vivo* hair growth effects of these mechanistically similar compounds are due exclusively to an effect on the hair follicle or whether they involve to some degree dilation of the scalp vasculature.

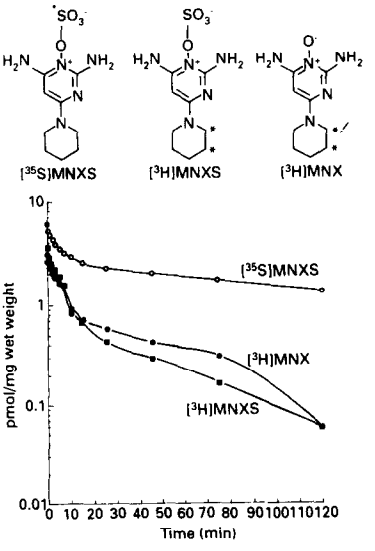

Fig. 3. Washout efflux of radiolabeled minoxidil sulfate (MNXS) and minoxidil (MNX) from intact rabbit mesenteric artery. The three radiolabeled molecules used are depicted in the top panel with * indicating positions of the radioisotope. The bottom panel presents a semilogarithmic plot showing the time course of washout efflux. The data are presented as the amount of drug (picomoles per milligram of wet weight) associated with the tissue at a given time point. Reprinted with permission from Ref. 35 (Meisheri KD, Oleynek JJ and Puddington L, Role of protein sulfation in vasodilation induced by minoxidil sulfate, a K^+ channel opener. *J Pharmacol Exp Ther* **258**: 1091–1097, 1991). Copyright (1991) American Society for Pharmacology and Experimental Therapeutics.

Role of protein sulfation in the cellular actions of minoxidil sulfate

The mechanism(s) by which potassium channel openers activate ATP-sensitive K^+ channels remains to be elucidated. The available data suggest that minoxidil sulfate is likely to be distinct in this respect. Recently we experimentally pursued the question as to why minoxidil sulfate, but not minoxidil, is an active relaxant of vascular smooth muscle *in vitro* [35]. We also wanted to understand why, unlike other K^+ channel openers (pinacidil or cromakalim), the vascular actions of minoxidil sulfate are quite long lasting. In a series of experiments using radiolabeled molecules, it was shown that the behavior of minoxidil sulfate is intrinsically different from that of minoxidil. As presented in Fig. 3, segments of intact rabbit mesenteric artery exposed to ^{35}S-labeled minoxidil sulfate retained the radiolabel. In contrast, retention of radiolabel was not detected with either ^3H-labeled minoxidil sulfate (label on the piperidine ring) or ^3H-labeled minoxidil. This was our first indication that the sulfate moiety from minoxidil sulfate was closely associated with the vascular tissue. Subsequent experiments demonstrated that smooth muscle proteins were acceptors of [^{35}S]sulfate from ^{35}S-labeled minoxidil sulfate. For this study, rabbit mesenteric artery rings were incubated with ^{35}S-labeled minoxidil sulfate for various times (2–30 min), and then SDS-solubilized proteins were resolved by SDS–PAGE. A fluorogram of the dried gel, presented in Fig. 4, showed a preferential ^{35}S-labeling of a 116 kDa protein. Additional experiments described in Meisheri *et al.* [35] demonstrated that the preferential labeling of the 116 kDa protein was temporarily related to the time course of vascular relaxation produced by minoxidil sulfate. An additional protein at 43 kDa, resembling smooth muscle α-actin, was also an acceptor of [^{35}S]sulfate from minoxidil sulfate (see Fig. 4; also see Fig. 7). However, unlike the labeling of the 43 kDa protein, the labeling of the 116 kDa protein was disproportionately greater than its relative abundance in total artery proteins, suggesting that the 116 kDa protein was a preferred acceptor of sulfate from minoxidil sulfate. These and other additional observations described in Ref. 35 led us to hypothesize that the minoxidil sulfate-induced, non-enzymatic covalent protein sulfation, observed under relevant pharmacological conditions, is critical for K^+ channel activation and vasodilation mediated by minoxidil sulfate. This covalent protein sulfation

* Meisheri KD, Taylor CJ and Cipkus LA, The mechanisms of vascular smooth muscle relaxing effects of minoxidil sulfate. In: *Smooth Muscle Function Symposium Proceedings: Official Satellite Symposium of the XXX International Physiology Congress, Banff, Canada, 1986*, p. 114.

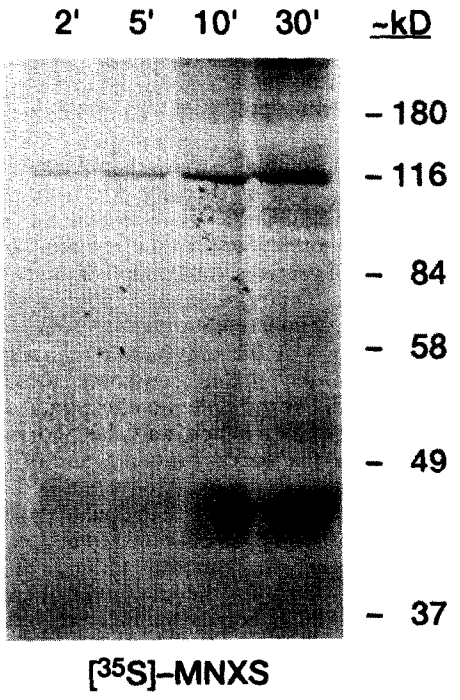

Fig. 4. Fluorogram of an SDS gel showing a time course (2–30 min) of ^{35}S-labeled minoxidil sulfate (MNXS) -induced protein sulfation in intact mesenteric artery. Preferential time-dependent labeling of a protein of approximately 116 kDa is evident. Labeling of a 43 kDa protein and less dominant labeling of other proteins are also observed. Reprinted with permission from Ref. 35 (Meisheri KD, Oleynek JJ and Puddington L, Role of protein sulfation in vasodilation induced by minoxidil sulfate, a K^+ channel opener. *J Pharmacol Exp Ther* **258**: 1098–1991). Copyright (1991) American Society for Pharmacology and Experimental Therapeutics.

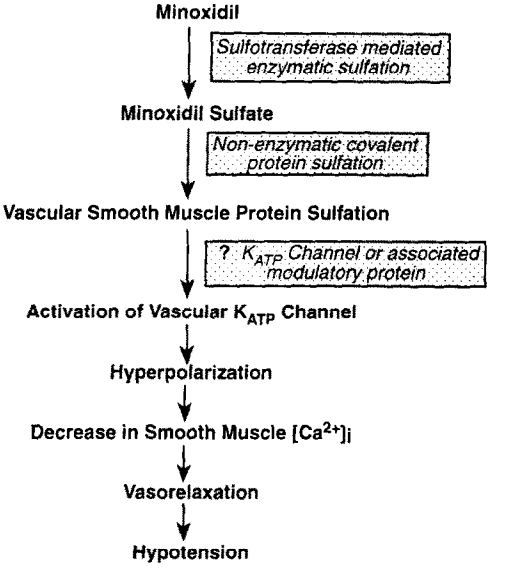

Fig. 5. Schematic diagram depicting the known and the proposed sequence of events in the actions of minoxidil. This scheme incorporates the majority of experimental observations made, and provides a framework for future experiments (see text for details).

could also account for the long-lasting relaxation and hyperpolarizing effects of minoxidil sulfate *in vitro* [35, 36] and of minoxidil *in vivo* [6].

Taken together, these observations allow us to present a schema, as shown in Fig. 5, that summarizes the known and proposed sequence of events in the actions of minoxidil. This scheme incorporates the majority of unique experimental observations reported for minoxidil and minoxidil sulfate, in particular for enzymatic sulfation to activate minoxidil, and for non-enzymatic protein sulfation to activate K^+ channels. Although certain aspects of this scheme remain hypothetical, it provides a framework for further experiments to identify protein(s) critical for the modulation of K^+ channels by minoxidil sulfate, and possibly other K^+ channel openers.

We have also examined the other important target tissue of minoxidil sulfate's action, the hair follicle, to determine if substrates of minoxidil sulfate-induced protein sulfation were similar to those in mesenteric vascular smooth muscle. Intact scalp follicles were dissected from human skin and incubated with ^{35}S-labeled minoxidil sulfate. Figure 6 shows that, similar to rabbit mesenteric artery, ^{35}S-labeling of 116 and 43 kDa proteins was also observed in hair follicles. The 116 kDa protein was localized in the dermal papilla component of the follicle in labeling studies of microdissected scalp follicles and cultured dermal papilla cells. As shown in Fig. 7, two-dimensional gel electrophoretic analysis of proteins from rabbit mesenteric artery and rat dermal papilla cells showed that the ^{35}S-labeled 116 kDa proteins had similar isoelectric points. We speculate that the temporal correlation observed between vasorelaxation and preferential sulfation of the 116 kDa protein in smooth muscle may also imply significance to sulfation in the mechanism by which minoxidil promotes hair growth. This speculation is based not only on what appears to be a similar substrate for sulfation by minoxidil sulfate in the two target tissues, but also on the experimental evidence that the enhanced K^+ conductance mechanism appears to be common to both therapeutic actions of minoxidil [27, 34].

Protein sulfation and K^+ channel activation

The concept of modulating ionic permeability of the cell membrane and thus cell function via drug-induced chemical sulfation of proteins is unorthodox and unprecedented. Thus, it requires further careful experimental analysis and novel approaches. Unlike other ion channel proteins the ATP-sensitive K^+ channel protein has not been identified and characterized. Success in this area will allow study of whether the purified channel protein can be sulfated by minoxidil sulfate and result in the expected change in its function.

The lack of sulfatase enzymes that remove sulfate

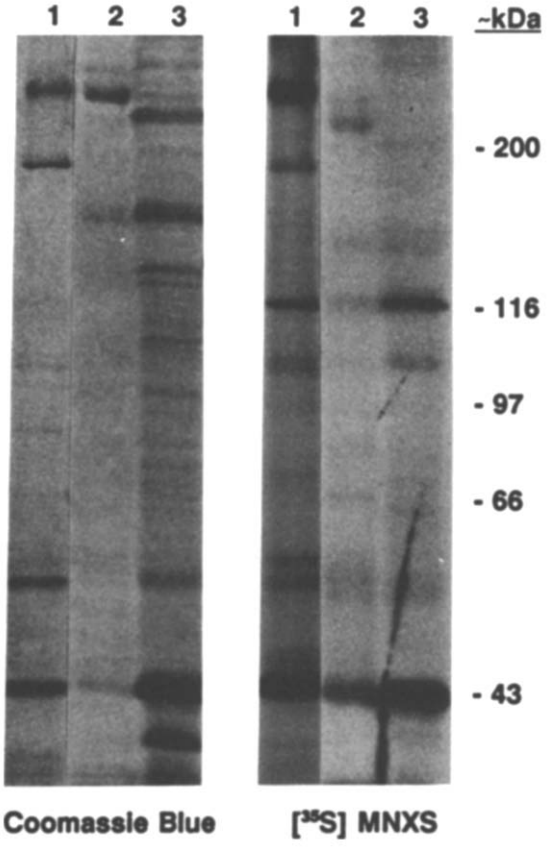

Fig. 6. Labeling of mesenteric artery, hair follicle, and dermal papilla cell proteins by [^{35}S]minoxidil sulfate ([^{35}S]-MNXS). Rat dermal papilla cells from passage 2 (lane 1), human hair follicles (lane 2), and rabbit mesenteric artery rings (lane 3) were incubated with 200 μCi/mL [^{35}S]MNXS (800 Ci/mmol) for 2 hr (1 and 2) or 15 min (3). Tissues were ground to powder in liquid nitrogen, and proteins solubilized in 7.2% SDS were subjected to electrophoresis on 10% polyacrylamide gels. Gels were stained with Coomassie Blue and then processed for fluorography. Note the labeling of two proteins of approximately 116 and 43 kDa in mesenteric artery and the dermal papillar component of the follicle.

moieties from proteins in eukaryotic cells makes non-enzymatic covalent protein sulfation an attractive strategy for effecting K^+ permeability of the plasma membrane. The resultant activation of substrate proteins by minoxidil sulfate would be irreversible, essentially until the protein was degraded, and new protein synthesized. This is unlike other post-translational modifications of proteins such as phosphorylation, glycosylation, or fatty acylation, all of which are constant targets of attack by enzymes capable of catalyzing their removal or modification. The known effects of sulfate addition on protein function have been best characterized for substrates of tyrosylprotein sulfotransferase (TPST). TPST is an integral membrane protein that catalyzes the addition of sulfate from phosphoadenosyl-phosphosulfate to specific tyrosine residues in the lumen of the trans Golgi complex [37]. Tyrosine sulfation has been shown to affect the biological activity of peptide hormones, such as cholecystokinin, whose hormonal activity is dependent upon sulfation of the tyrosine residue [38]. Sulfated tyrosine residues can also protect regions of proteins from proteolytic degradation, for example secretogranin I and II [39]. It is interesting that a capacity for overlapping substrate specifications has been shown for TPST and autophosphorylating tyrosine protein kinase using a variety of peptides, for example those derived from α-tubulin, cholecystokinin-1, and pp60$^{C\text{-}SIC}$ [40]. Therefore, in intact cells, the compartmentation of enzyme activity is the only factor that restricts modification of the shared consensus sites, i.e. sulfation by TPST is the only modification at a site that occurs in the Golgi lumen, and phosphorylation by protein kinase is the only modification of a site that occurs in the cytoplasm.

The ability of minoxidil sulfate to donate its sulfate moiety to at least one protein known to be localized in the cytoplasm, i.e. smooth muscle α-actin, provides a mechanism for the cross-over of protein sulfation into cellular compartment where protein kinases are located. It can be speculated that if the consensus sites for modification by minoxidil sulfate resemble those shared by TPST and tyrosine protein kinases, the substitution of sulfate for phosphate would result in an irreversible, rather than transient, change in protein conformation. Since it is known that phosphorylation is involved in the activation of several ion channels, including K^+ channels, it is conceivable that the appropriate sulfation of cytoplasmic domains of K^+ channels or associated proteins would result in channel opening.

It should be pointed out that although linkages generated between sulfate or phosphate and hydroxyl groups on tyrosine result in formation of the respective ester, addition of sulfate to proteins by minoxidil sulfate is not believed to be limited to tyrosine residues. Other amino acids which form stable sulfate conjugates *in vitro* include serine, threonine, and cysteine, although no evidence for the existence of these modified amino acids in eukaryotic cells has been obtained [37]. It is interesting that a -SH group, possibly contributed by a cysteine residue, has been identified that appears to be significant for gating of the ATP-sensitive K^+ channels [41]. Chemically, minoxidil sulfate is considered to be a very reactive molecule with respect to its ability to donate its sulfate to nucleophiles such as proteins. Thus, in addition to being capable of sulfating proteins in a compartment of the cell where it does not normally occur, minoxidil sulfate should have additional capacity to modify proteins in ways that have never been observed to occur *in vitro* or *in vivo*. A preliminary study of peptides derived from angiotensin II showed that N-termini and histidine residues were the predominant sites of sulfation by minoxidil sulfate *in vitro* [42].

Summary and future directions

This review has highlighted two major areas of interest for minoxidil research. One has been the characterization of minoxidil sulfotransferase, an enzyme responsible for the bioactivation of minoxidil.

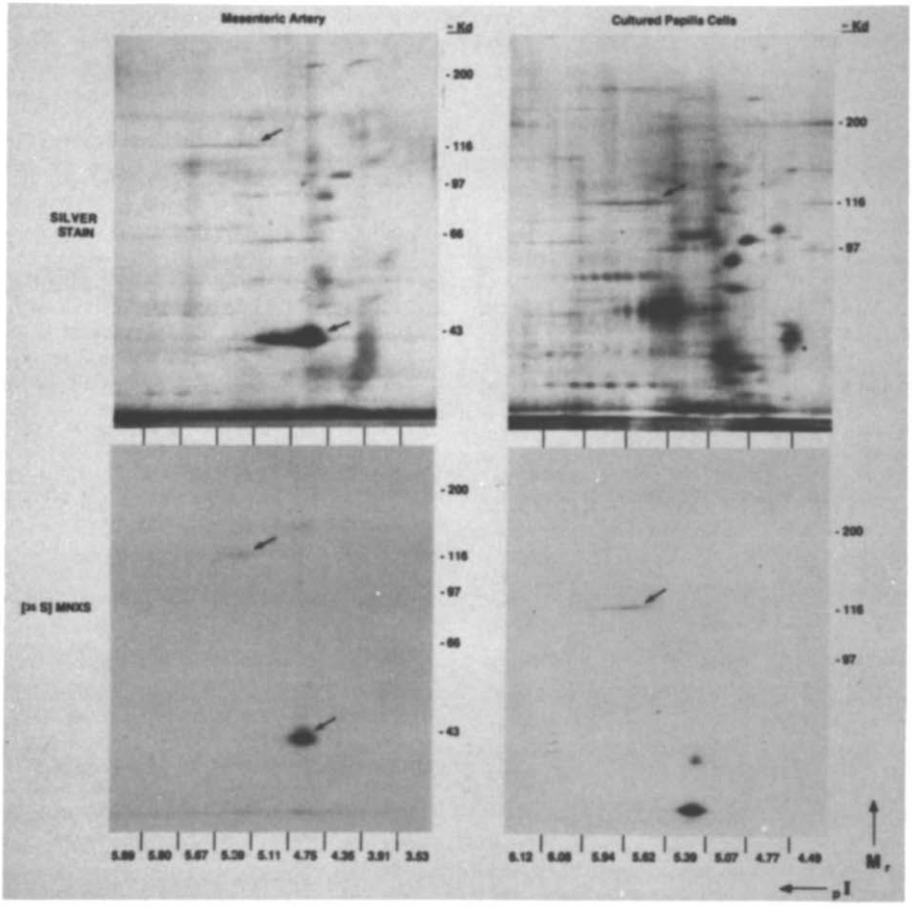

Fig. 7. Two-dimensional electrophoresis analysis of proteins from mesenteric artery and dermal papilla cells labeled by [^{35}S]minoxidil sulfate ([^{35}S]MNXS). Tissues were labeled as described in Fig. 6. Proteins were solubilized in 8 M urea and subjected to isoelectric focusing. Separation in the second dimension was performed on 10% polyacrylamide gels. Gels were silver stained, then destained, and processed for fluorography. Note that the 116 kDa ^{35}S-sulfate acceptor proteins in the two tissues had similar isoelectric properties.

As pointed out, the molecular characterization of this enzyme will be of interest, since it may provide a rationale for the quantitatively differential responses to minoxidil in humans, particularly for the hair growth stimulating effect of topical minoxidil. The second area has been the description of the cellular and molecular mechanisms involved in the actions of minoxidil sulfate. The latter area, particularly the role of covalent protein modification through non-enzymatic sulfation in ion channel modulation, may prove to be challenging and yet quite interesting. It should be noted that the mechanisms through which various structurally different K^+ channel openers activate the ATP-sensitive K^+ channel remain to be elucidated. Biochemical interactions between K^+ channel openers and glyburide, a potent blocker of the ATP-sensitive K^+ channel, have been described recently [43]. Once the protein(s) constituting the ATP-sensitive K^+ channel is identified and characterized via biochemical and molecular biology techniques, it will be interesting to study if these proteins are substrates for the actions of minoxidil sulfate, and to study their associations with the 116 kDa preferred acceptor of sulfate from minoxidil sulfate. If the history of minoxidil's research is any indication, these future explorations will also prove quite rewarding.

Acknowledgements—We wish to dedicate this article to Dr. Gerald R. Zins. Dr. Zins played a pioneering role in the early research of minoxidil. Furthermore, he has, in his capacity as the Director of Cardiovascular Diseases Research and later as the Director of Hair Growth Research, provided an intellectually stimulating environment and has encouraged us to experimentally pursue interesting questions in this field. We thus gratefully acknowledge here his *direct* as well as *indirect* contributions. We thank Kay Knight for her technical help during experiments with hair follicles. We also thank Drs. Jackson B. Hester and Donald W. DuCharme for constructive comments regarding this manuscript.

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