Minoxidil-Induced Hair Growth is Mediated by Adenosine in Cultured Dermal Papilla Cells: Possible Involvement of Sulfonylurea Receptor 2B as a Target of Minoxidil

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The mechanism by which minoxidil, an adenosine-triphosphate-sensitive potassium channel opener, induces hypertrichosis remains to be elucidated. Minoxidil has been reported to stimulate the production of vascular endothelial growth factor, a possible promoter of hair growth, in cultured dermal papilla cells. The mechanism of production of vascular endothelial growth factor remains unclear, however. We hypothesize that adenosine serves as a mediator of vascular endothelial growth factor production. Minoxidil-induced increases in levels of intracellular Ca^{2+} and vascular endothelial growth factor production in cultured dermal papilla cells were found to be inhibited by 8-sulfophenyl theophylline, a specific antagonist for adenosine receptors, suggesting that dermal papilla cells possess adenosine receptors and sulfonylurea receptors, the latter of which is a well-known target receptor for adenosine-triphosphate-sensitive potassium channel openers. The expression of sulfonylurea receptor 2B and of the adenosine A1, A2A, and A2B receptors was detected in dermal papilla cells by means of reverse transcription polymerase chain reaction analysis. In order to determine which of the adenosine receptor subtypes contribute to minoxidil-induced hair growth, the effects of subtype-specific antagonists for adenosine receptors were investigated. Significant inhibition in increase in intracellular calcium level by minoxidil or adenosine was observed as the result of pretreatment with 8-cyclopentyl-1,3-dipropylxanthine, an antagonist for adenosine A1 receptor, but not by 3,7-dimethyl-1-propargyl-xanthine, an antagonist for adenosine A2 receptor, whereas vascular endothelial growth factor production was blocked by both adenosine A1 and A2 receptor antagonists. These results indicate that the effect of minoxidil is mediated by adenosine, which triggers intracellular signal transduction via both adenosine A1 and A2 receptors, and that the expression of sulfonylurea receptor 2B in dermal papilla cells might play a role in the production of adenosine. ***Key words: adenosine receptor/ATP-sensitive potassium channel/hypertrichosis/intracellular calcium/vascular endothelial growth factor. J Invest Dermatol 117:1594–1600, 2001***

Minoxidil has been used for patients with severe hypertension who were refractory to other treatments (Dargie *et al*, 1977). The opening of the adenosine triphosphate (ATP) sensitive potassium channel (K_{ATP} channel), followed by the generation of membrane potential, results in a reduction of intracellular calcium ($[Ca^{2+}]i$) levels and in vascular smooth muscle relaxation, which is generally thought to contribute to this hypotensive effect (Andersson, 1992). In addition to this effect, minoxidil (Earhart *et al*, 1977; Burton and Marshall, 1979), pinacidil, diazoxide, and other K_{ATP} channel openers (Burton *et al*, 1975; Goldberg, 1988) have been reported to cause hypertrichosis in humans, though the precise mechanisms by which these drugs induce hair growth remains unclear. It is known, however, that despite their diverse chemical structures they share a common feature; namely, their actions of K_{ATP} channel opening. Dermal papilla cells (DPC), which reside at the base of hair follicles, were highly implicated as the source of regulatory stimuli of hair growth and development. Therefore, K_{ATP} channel opening in DPC has been suggested as an important mechanism in terms of the ability of DPC to stimulate hair growth (Buhl *et al*, 1993).

K_{ATP} channels comprise a sulfonylurea receptor (SUR), a member of the ATP-binding cassette transporter, and an inward rectifier potassium channel (Aguilar-Bryan *et al*, 1998). SUR is considered to be a direct receptor for the K_{ATP} channel openers, which could function as a pump for ATP (Awqati, 1995; Schwanstecher *et al*, 1998). Thus far, however, no studies have been conducted to determine whether SUR is expressed in DPC. Concerning the activity of the K_{ATP} channel in cultured DPC, minoxidil failed to induce an increase in K^+ permeability, either in the K_{ATP} channel or in the Ca^{2+}-activated K^+ channels (Nakaya *et al*, 1994; Hamaoka *et al*, 1997), suggesting that minoxidil-induced hair growth may be due to other mechanisms that are independent of potassium channel opening.

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Abbreviations: A1 (2A, 2B) R, adenosine A1 (A2A, A2B) receptor; CPX, 8-cyclopentyl-1,3-dipropylxanthine; DMPX, 3,7-dimethyl-1-propargyl-xanthine; DPC, dermal papilla cell; K_{ATP} channel, ATP-sensitive potassium channel; 8-SPT, 8-sulfophenyl theophylline; SUR, sulfonylurea receptor; VEGF, vascular endothelial growth factor.

Table I. Primers used in this study

Primer		Sequence	Database accession no.	Annealing temp.	Product size
AR1	sense	5′-TGCTGAAGGCGTCGAGGTGT-3′	GenBank X68485	60°C	765 bp
	antisense	5′-GCTTGCGGATTAGGTAGAAGAC-3′			
A2AR	sense	5′-CATCATGGGCTCCTCGGT-3′	GenBank X68486	60°C	633 bp
	antisense	5′-CTCTCCATCTGCTTCAGC-3′			
A2BR	sense	5′-CAACTGCACAGAACCCTG-3′	GenBank X68487	55°C	479 bp
	antisense	5′-GGCAGAGAAGATACCTGGAG-3′			
SUR1	sense	5′-CTCCAACTACCTCAACTGGATGC-3′	GenBank L78207	55°C	922 bp
	antisense	5′-TATGGGCAGGGTCCGAATGTGG-3′			
SUR2A	sense	5′-CCACAAGAAGGGGAGATCAAGA-3′	DDBJ AF061317.1 (exon32 of SUR2)	55°C	662 bp
	antisense	5′-CACTCCACTAAAATACCCTCAGAA-3′	DDBJ AF061323.1 (SUR2A specific exon38)		
SUR2B	sense	5′-CAAATGCACAGATGACAGACT-3′	DDBJ AF061620.1 (exon35 of SUR2)	52°C	411 bp
	antisense	5′-TCTGCGCGAACAAAAGAAGC-3′	DDBJ AF061324 (SUR2B specific exon38)		

Lachgar *et al* (1998) reported that minoxidil stimulates the production of growth factors such as vascular endothelial growth factor (VEGF) in cultured DPC, and that these growth factors might promote hair growth. The mechanism by which minoxidil increases the production of VEGF is not known, however.

Among the possible candidates for stimulators of VEGF production, adenosine seems to cause essential effects on a variety of cellular functions by triggering intracellular signal transduction and subsequent VEGF secretion in an autocrine or paracrine manner (Shryock and Belardinelli, 1997). With regard to possible sources of adenosine, ATP, which is secreted either through the ATP-binding cassette transporter or other currently unknown pathways (Awqati, 1995), is rapidly converted to adenosine by ecto-ATPase.

The aim of this study was to address whether minoxidil mediated by adenosine is capable of increasing $[Ca^{2+}]i$ levels and VEGF production. Our findings show that minoxidil caused an increase in $[Ca^{2+}]i$ levels and VEGF production in cultured DPC, and that these increases were inhibited by 8-sulfophenyl theophylline (8-SPT), a specific antagonist for adenosine A1/A2 receptors (A1R, A2R). This result suggests the presence of both adenosine receptors and a direct receptor for minoxidil, possibly SUR, in DPC. We were also able to confirm the gene expression of adenosine receptors and SUR in DPC by means of reverse transcription polymerase chain reaction (RT-PCR) analysis and subsequent sequence determination of the PCR products.

MATERIALS AND METHODS

Cell culture DPC were cultured as described previously (Messenger, 1984). Dermal papillae were enucleated from excised hair follicles with the intact bulbous portion, and then cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 12% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2/air. DPC were subcultured after they had grown out from the papillae and achieved subconfluence.

$[Ca^{2+}]i$ measurements Fura-2/acetoxymethyl ester (Wako Pharmaceuticals, Tokyo, Japan) was used for the measurement of $[Ca^{2+}]i$ concentration. Cells were cultured on 13 mm cover glasses (Matsuura Industry, Tokyo, Japan) and washed with modified normal Tyrode's solution, which contained 140 mM NaCl, 4.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM 3-morpholinopropanesulfonic acid, and 10 mM glucose (pH 7.2–7.4). Fura-2/acetoxymethyl ester (4 μM) was added and the mixture was incubated for 30 min at 37°C. The cover glass was then transferred to the circulated temperature-controlled chamber of the analysis system, an ARGUS-50 (Hamamatsu Photonics, Hamamatsu, Japan), and the test solution was perfused. Any emitted fluorescence in DPC on the cover slips was measured using a fluorescence spectro-microscope (excitation, 340/380 nm; emission, 510 nm). The results were expressed as the ratio of the fluorescence recorded at 340 nm and 380 nm. Changes in this ratio were detected when minoxidil [in this study, minoxidil sulfate was used as the active form of minoxidil, and was synthesized according to the method described by Newgreen *et al* (1990)] or adenosine (Wako) was added to the test solution alone or together with one of the following: the antagonist for A1R/A2R, 8-SPT (Sigma-Aldrich Chemie, Steinheim, Germany); a specific antagonist for A1R, 8-cyclopentyl-1,3-dipropylxanthine (CPX) (Sigma); or a specific antagonist for A2R, 3,7-dimethyl-1-propargyl-xanthine (DMPX) (Sigma).

Secretion of VEGF Secreted VEGF from cultured DPC was determined using a kit for the sandwich enzyme-linked immunosorbent assay (ELISA) of human VEGF (Genzyme/TECHNE, Minneapolis, MN). After reaching subconfluence, DPC were cultured in a 35 mm dish with 1% fetal bovine serum for 24 h. Adenosine or minoxidil was added to 90% confluent cultures of DPC alone or together with antagonists for adenosine receptors. Culture media were then collected for measurements. Because adenosine and minoxidil are not stable, these compounds (10 μl of 10^{-5} M solution) were added to the culture media at 6 h intervals. After 24 h, the culture media were collected in order to measure VEGF.

A monoclonal antibody specific for VEGF was precoated onto a microplate. The collected supernates were pipetted into the wells, where VEGF in the sample was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody, which is specific for VEGF, was added to the wells. Following a wash to remove any unbound antibody–enzyme reagent, a substrate solution was added to the wells, and the colors developed were proportional to the amount of VEGF bound in the initial step. Color development was stopped and the intensity of the color was measured.

RT-PCR and sequence analysis After reaching subconfluence in DMEM medium, DPC were washed with phosphate-buffered saline (PBS). Total RNA was extracted from the cells using the acid guanidinium–phenol–chloroform method (Chomczynski and Sacchi, 1987). To determine expression of SURs and adenosine receptors in DPC, the reverse transcription of 5 μg of total RNA was performed using the Superscript Preamplification System for First Strand cDNA Synthesis according to the manufacturer's instructions (Life Technologies, Rockville, MD). The PCR was carried out according to a previously described method (Rappolee *et al*, 1988). In brief, the reaction was performed in 50 μl of reaction mixture, containing 2 μl of reverse transcription mixture prepared by the preamplification system described above, 1 μl of each gene-specific primer (10 μM), and 0.5 μl of 5 units per μl "long and accurate" (LA) taq DNA polymerase (Takara Biotechnology, Kyoto, Japan). After an initial denaturation step for 1 min at 94°C, the DNA was amplified for 30 cycles at 94°C for 30 s, various annealing temperatures for 30 s, and at 72°C for 2 min on a DNA cycler, followed by electrophoresis. Six pairs of specific oligonucleotide primers for each of the receptors were used, as shown in **Table I**, and were synthesized by Gibco BRL (Tokyo, Japan). Specific primers for human GAPDH (5′-CCACCCATGGCAAATTCC-ATGGCA-3′, sense, and 5′-TCTAGACGGCAGGTCAGGTCCACC-3′, antisense) were purchased from Clontech (Tokyo, Japan). The

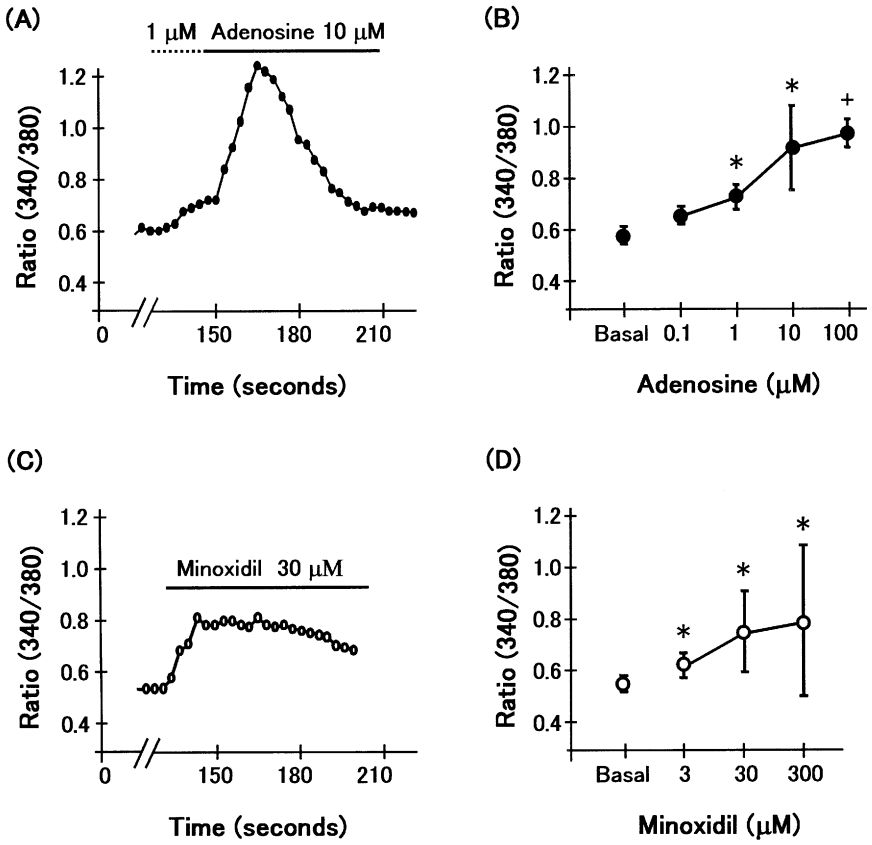

Figure 1. Changes in $[Ca^{2+}]i$ levels caused by adenosine and minoxidil. The test solution containing adenosine (10 μM) caused a significant increase in $[Ca^{2+}]i$ levels in cultured DPC, as evidenced by the appearance of an initial peak (*A*). The test solution containing minoxidil (30 μM) alone also caused a significant increase in $[Ca^{2+}]i$ levels (*C*), at a slower rate than that with adenosine, followed by a steady increase over time. Concentration-dependent elevations of $[Ca^{2+}]i$ are shown in (*B*) and (*D*), respectively. The increase was significant at 100 μM in adenosine and at 300 μM in minoxidil ($n > 7$ at each point). *, +, $p < 0.05$ and $p < 0.01$ *vs* control, respectively.

amplified DNA fragments were subcloned into a pT7Blue cloning vector (TaKaRa Shuzo, Shiga, Japan) and sequenced by means of a BigDye terminator cycle sequencing kit in an ABI 310 genetic analyzer (ABI PRISM 310, PE Applied Biosystems).

Statistical analysis Student's *t* test was used to determine the significance of differences in findings before and after the application of adenosine receptor antagonist. p-values of less than 0.05 were considered significant.

RESULTS

Changes in $[Ca^{2+}]i$ levels by minoxidil and adenosine Based on the fact that intracellular calcium is a ubiquitous second messenger, which regulates a wide range of cellular processes associated with a variety of fundamental cellular functions (Toescu, 1995), including VEGF gene expression (Mukhopadhyay and Akbarali, 1996), we first examined the effect of adenosine and minoxidil on $[Ca^{2+}]i$ levels in cultured DPC. As shown in **Fig 1**(***A***), adenosine (10 μM) initially increased the levels of $[Ca^{2+}]i$ (ratio of 340/380 nm) in cultured DPC, followed by a small sustained elevation of $[Ca^{2+}]i$. The relation between the dose of adenosine and the elevation of the $[Ca^{2+}]i$ peak is shown in **Fig 1**(***B***).

Minoxidil (30 μM) caused a gradual increase in $[Ca^{2+}]i$ levels over time in cultured DPC (**Fig 1*C***), although the rate of elevation was slow. The dose-dependent increase in $[Ca^{2+}]i$ levels by minoxidil is shown in **Fig 1**(***D***). In the absence of extracellular Ca^{2+}, neither minoxidil nor adenosine caused an increase $[Ca^{2+}]i$ levels (data not shown).

In order to determine whether the minoxidil-induced increase in $[Ca^{2+}]i$ levels is mediated by adenosine, the effect of an A1R/A2R antagonist, 8-SPT, was examined (**Fig 2**). In the presence of 1 μM 8-SPT alone, the fluorescence ratio of 340/380 nm was not altered, although it was increased at higher concentrations of 8-SPT. Therefore, we used 1 μM 8-SPT in this experiment. The increase in $[Ca^{2+}]i$ levels, which was induced by the presence of 10 μM adenosine or 30 μM minoxidil, was almost completely blocked by 1 μM 8-SPT (**Fig 2*A***, ***B***, *open circles*).

Changes in VEGF secretion caused by minoxidil and adenosine **Figure 3** shows the secretion of VEGF from cultured DPC by minoxidil or adenosine. Minoxidil, at a concentration of 30 μM, and adenosine, at a concentration of 10 μM, significantly increased the production of VEGF (both $p < 0.05$), and these increases were suppressed in the presence of 1 μM 8-SPT (both $p < 0.05$). 1 μM 8-SPT alone had no effect on the production of VEGF.

Gene expression of adenosine receptors in DPC The increases in $[Ca^{2+}]i$ levels were inhibited by the A1R/A2R antagonist, suggesting the presence of adenosine receptors in DPC. We therefore examined whether the genes encoding adenosine receptors were expressed in DPC, and, if so, which subtype is expressed. RT-PCR reactions were performed using specific primers for the A1R, A2AR, and A2BR, followed by agarose gel electrophoreses of the products. The expected sizes of the specific PCR products were 765 bp for A1R, 633 bp for A2AR, and 479 bp for A2BR (**Fig 4**). DPC showed bands corresponding to the expected size of all three of these subtypes of adenosine receptors. Sequence analyses of these products were performed after subcloning them into the pT7Blue cloning vector. All of the sequences were identical with those found in the DDBJ database (data not shown).

Effect of subtype-specific adenosine receptor antagonist on minoxidil-induced increase in $[Ca^{2+}]i$ levels and VEGF production Based on the finding that gene expression of all of A1R, A2AR, and A2BR were detected in DPC, we focused attention on which of these receptors contributes to the minoxidil-induced increase in $[Ca^{2+}]i$ levels and VEGF production. The receptor subtypes involved were characterized using the selective receptor antagonists CPX and DMPX, selective antagonists for A1R and A2R, respectively. The increase in $[Ca^{2+}]i$ levels, as

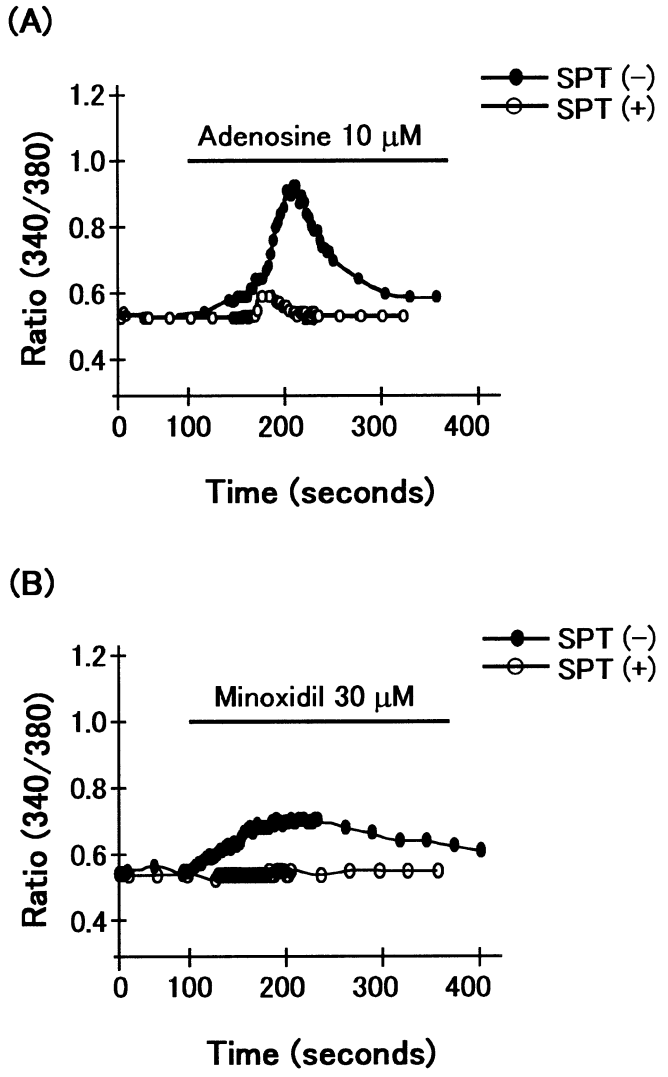

Figure 2. Effects of a specific antagonist for adenosine receptors on the minoxidil-induced increase in $[Ca^{2+}]i$ levels. The effect of the antagonist for A1R/A2R, 8-SPT, was tested on the adenosine-induced increase in $[Ca^{2+}]i$ levels (*A*). In the presence of 8-SPT (*open circles*), the increase in $[Ca^{2+}]i$ levels by adenosine (*closed circles*) was blocked ($n = 8$). The effect of 8-SPT was tested on the minoxidil-induced increase in $[Ca^{2+}]i$ levels (*B*). In the presence of 8-SPT (*open circles*), minoxidil ($n = 10$) did not cause an increase in $[Ca^{2+}]i$ levels, compared with the increase caused by the test solution containing minoxidil alone (*closed circles*).

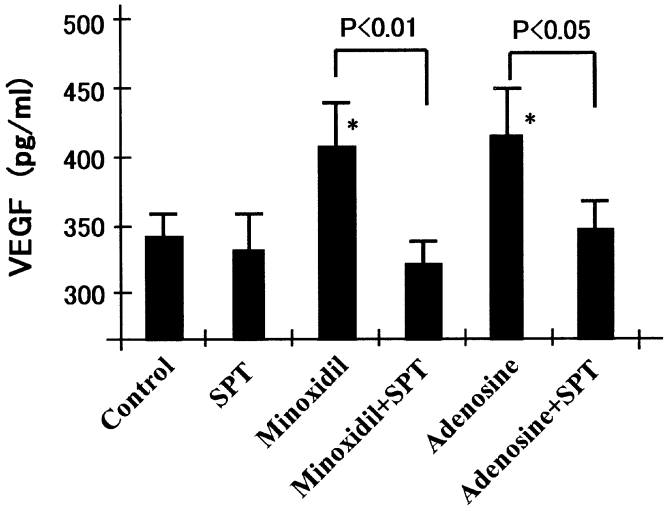

Figure 3. Production of VEGF by adenosine or minoxidil in cultured DPC. The addition of 10 μM adenosine ($n = 8$) or 30 μM minoxidil ($n = 8$) for 24 h increased the production of VEGF. These increases were significantly suppressed in the presence of 1 μM 8-SPT, an A1R/A2R antagonist. 1 μM SPT alone did not cause a change in VEGF production in cultured DPC. *$p < 0.05$ *vs* control.

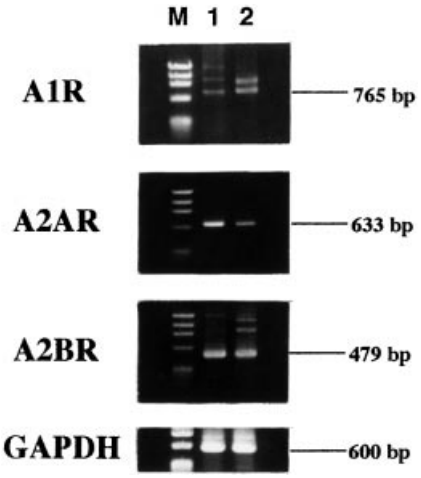

Figure 4. RT-PCR analyses confirmed the expression of adenosine receptors in DPC. cDNAs from DPC (*lane 2 s*) were amplified with primers specific for A1R, A2AR, and A2BR. *Lane 1 s* shows the PCR product of human vascular endothelial cells, which were used as a positive control. GAPDH mRNA was used as a quantitative RNA control. The sizes expected for the specific PCR products are shown on the right. M, marker.

induced by 10 μM adenosine (**Fig 5*A***, ***B***, *closed circles*) was almost completely blocked by 5 nM CPX (**Fig *5A***, *open triangles*), but not by 3 μM DMPX (**Fig 5*B***, *open squares*). Similar results were obtained when minoxidil was used in place of adenosine (**Fig 5*C***, *open triangles*, and **Fig 5*D***, *open squares*). To exclude the possibility of direct involvement of the adenosine receptor antagonist in the changes in $[Ca^{2+}]i$ levels, either 5 nM CPX or 3 μM DMPX alone was added to the DPC culture. In the presence of either of these, the fluorescence ratio of 340/380 nm was not altered (data not shown). To summarize, a significant inhibition in the increase in $[Ca^{2+}]i$ levels caused by adenosine (**Fig 5*E***) or minoxidil (**Fig 5*F***) was observed in the case of CPX (both $p < 0.01$), whereas no change was observed when DMPX was used.

We also tested the effects of CPX and DMPX on minoxidil-induced secretion of VEGF. In contrast, the increase in VEGF production induced by 10 μM adenosine or 30 μM minoxidil was inhibited by both 5 nM CPX (both $p < 0.05$) and 3 μM DMPX (both $p < 0.05$) (**Fig 6**). Neither 5 nM CPX nor 3 μM DMPX altered the production of VEGF.

Gene expression of SUR2B in DPC Based on the data presented above, we assume that the effect of minoxidil is mediated by adenosine in cultured DPC, which suggests the endogenous production of adenosine or a precursor thereof in response to minoxidil. Given that SUR might function as a pump for ATP (Awqati, 1995), the expression of SUR, a target receptor for K_{ATP} channel openers, was investigated in DPC. RT-PCR was performed using specific primers for SUR1, SUR2A, and SUR2B, followed by agarose gel electrophoreses of the products. Among these three subtypes, only the SUR2B product was detected at the expected size of 411 bp (**Fig 7**). Subsequent sequence analysis revealed that the sequence was identical to that submitted to the DDBJ database (data not shown).

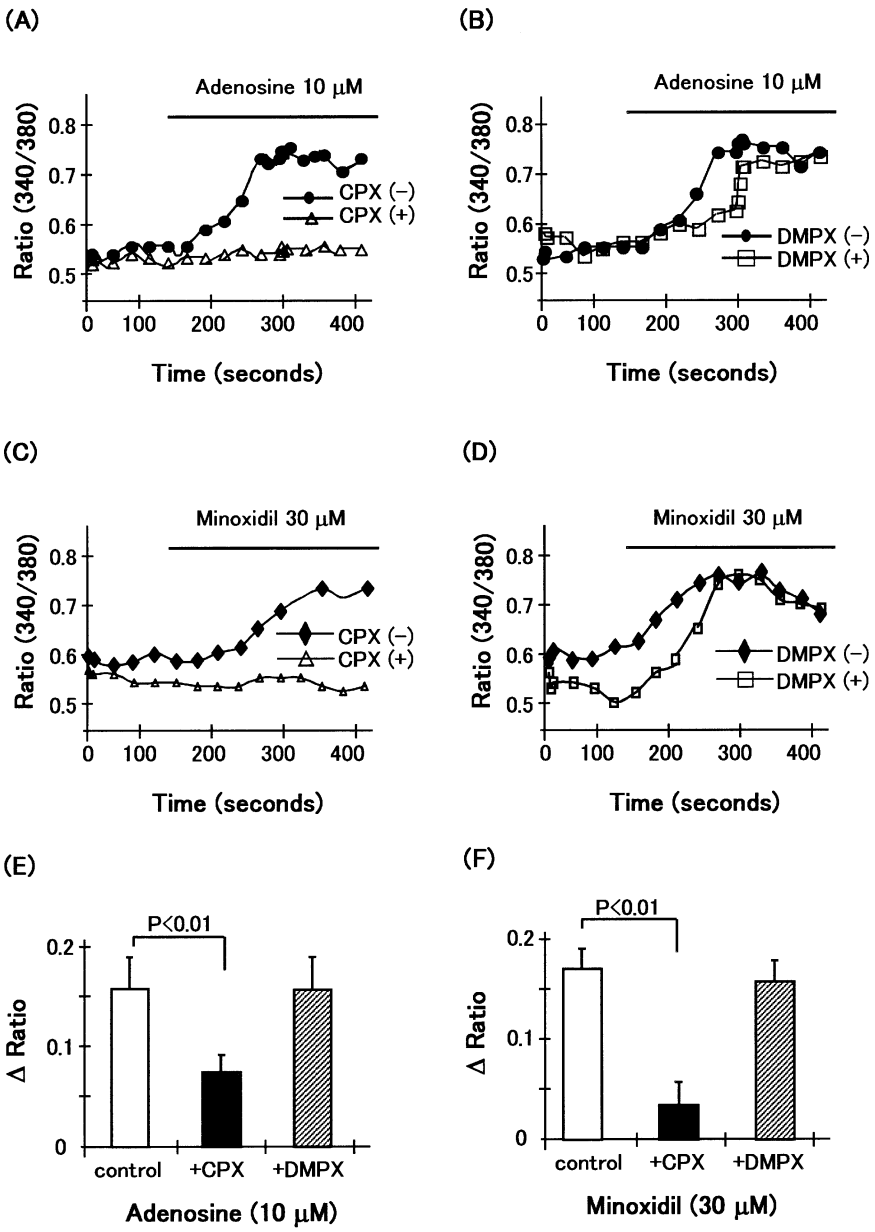

Figure 5. Effects of subtype-selective antagonists for adenosine receptors on the minoxidil-induced increase in $[Ca^{2+}]i$ levels. The increases caused by either adenosine ($n = 8$) or minoxidil ($n = 8$) were completely inhibited when 5 nM CPX, a selective antagonist for A1R, was added to the test solution (*open triangles*, *A* and *C*), but this was not the case when 3 μM DMPX, a selective antagonist for A2R, was added (*open squares*, *B* and *D*). (*A*) The test solution contained adenosine in the presence (*open triangles*) or absence (*closed circles*) of CPX; (*B*) adenosine in the presence (*open squares*) or absence (*closed circles*) of DMPX; (*C*) minoxidil in the presence (*open triangles*) or absence (*closed diamonds*) of CPX; (*D*) minoxidil in the presence (*open squares*) or absence (*closed diamonds*) of DMPX. Statistical analysis showed a significant inhibitory effect of CPX on both the adenosine-induced (*E*) and minoxidil-induced (*F*) increase in $[Ca^{2+}]i$ levels.

DISCUSSION

The elevation of $[Ca^{2+}]i$ and the production of VEGF are known to stimulate cell proliferation. This study showed that minoxidil increased both $[Ca^{2+}]i$ levels and VEGF production in cultured DPC in a manner similar to that of adenosine, and these increases were inhibited by an adenosine receptor antagonist. Thus, we hypothesize that the stimulation of DPC by minoxidil activated an adenosine/adenosine receptor signal transduction pathway (**Fig 8**). In support of this hypothesis, the presence of adenosine receptors in DPC was investigated, revealing the presence of all of the A1R, A2AR, and A2BR subtypes.

VEGF not only is a potent stimulator of vasodilation, microvascular hyperpermeability, and angiogenesis, but also serves as a multifunctional growth factor for a variety of cells. Lachgar *et al* (1996) reported that DPC express VEGF, and that VEGF acts on DPC as an autocrine growth factor. They (Lachgar *et al*, 1998) also found that the production of VEGF is promoted by minoxidil in cultured DPC, and concluded that minoxidil promotes hair growth via the induced VEGF. The molecular mechanisms governing VEGF production by minoxidil have not yet been clearly elucidated, however.

Adenosine stimulates VEGF production through the activation of cell surface adenosine receptors (Hashimoto *et al*, 1994; Takagi *et al*, 1996). This study has shown that minoxidil, like adenosine, has a positive effect on promoting $[Ca^{2+}]i$ and VEGF production, and that these effects are blocked by specific adenosine receptor antagonists, indicating that minoxidil-induced hair growth is mediated by adenosine. The gene expression of adenosine receptors in DPC confirmed the endogenous production of VEGF. The structure of adenosine receptors governs the various facets of subtype activity (Olah and Stiles, 2000). A functional subtype determination experiment in our study demonstrated that the receptors involved in the effect of increasing $[Ca^{2+}]i$ levels belong to the A1R subtype, and that, on the other hand, VEGF production could be activated via both A1R and A2R within the same DPC. These results indicate that DPC themselves possess multiple adenosine-dependent signaling pathways.

The opening of K_{ATP} channels has been considered to be a common mechanism for the action of minoxidil and a set of potassium channel openers (Buhl *et al*, 1993). Several investigators have described a beneficial effect of the K_{ATP} channel on cardioprotective vasorelaxation associated with adenosine receptors (Kato *et al*, 2000; Roscoe *et al*, 2000). There are three possibilities

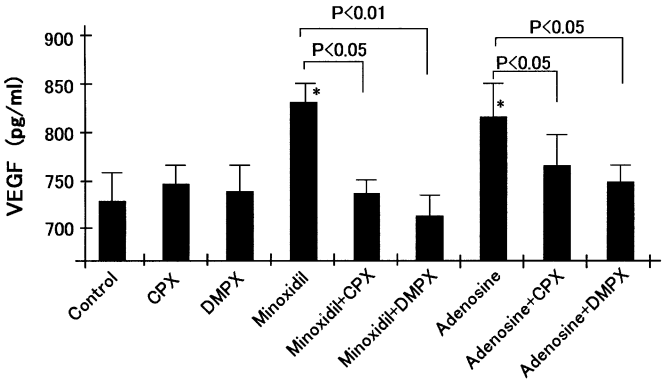

Figure 6. Effects of subtype-selective antagonists for adenosine receptors on the minoxidil-induced VEGF production. The increase in VEGF production caused by either adenosine ($n = 5$) or minoxidil ($n = 5$) was inhibited when 5 nM CPX or 3 μM DMPX was added to the test solution for 24 h of incubation. VEGF production induced by 10 μM adenosine or 30 μM minoxidil was inhibited by 5 nM CPX and 3 μM DMPX, respectively. Neither 5 nM CPX nor 3 μM DMPX had any effect on VEGF production in cultured DPC. *$p < 0.05$ *vs* control.

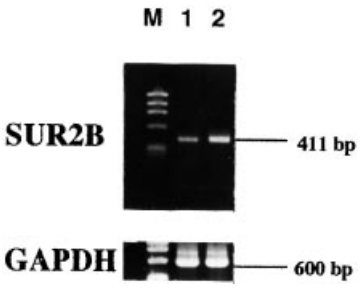

Figure 7. RT-PCR analysis confirmed the expression of SUR2B in DPC. cDNAs from DPC (*lane 2*) were amplified with primers specific for SUR2B. The PCR product of human aortic smooth muscle cell cDNA (*lane 1*) was used as a positive control and GAPDH mRNA was used as a quantitative RNA control. The size expected for the specific PCR product is shown on the right. M, marker.

regarding this process: (i) K_{ATP} channels could activate adenosine receptors directly, although no evidence is currently available to suggest that a binding site for K_{ATP} channel openers exists in adenosine receptors – thus far, SUR is considered to be a receptor for minoxidil, and to have a specific binding site for the K_{ATP} channel opener; (ii) K_{ATP} channels couple to adenosine receptors via mediators, such as G proteins (Kirsch *et al*, 1990). (iii) K_{ATP} channel openers act not only to open an inward rectifier potassium channel but also to promote the secretion of ATP either through SUR or via an unknown pathway (Awqati, 1995; Kitakaze *et al*, 1996). ATP is rapidly converted to adenosine by ecto-ATPase. Our results strongly indicate the possibility that a contribution by SUR has an effect on the minoxidil-activated adenosine signal transduction pathway.

Three subtypes of SURs have been recognized to date: pancreatic type (SUR1), the inhibition of which facilitates insulin secretion; cardiac type (SUR2A), which provides myocardial protection; and vascular smooth muscle type (SUR2B), which plays a role in vasorelaxation (Yokoshiki *et al*, 1998). Differential sensitivity to sulfonylureas or dizaoxide has been shown among these three isoforms (Aguilar-Bryan *et al*, 1998). This study has shown that SUR2B, but not SUR1 or SUR2A, is present in DPC, which is consistent with the report that minoxidil has a specific affinity for SUR2B (Schwanstecher *et al*, 1998). The expression of SUR2B in DPC provides a possible binding site to minoxidil and a potential mediator for adenosine production.

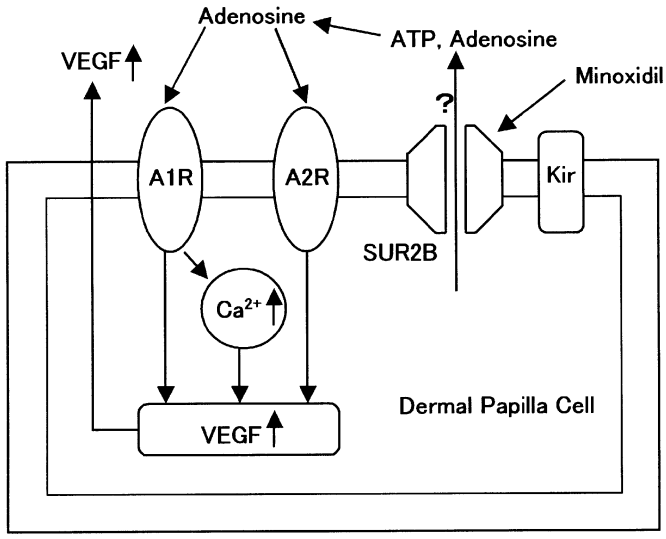

Figure 8. Proposed signaling pathway mediating minoxidil action in cultured DPC. The proposed pathway links minoxidil-induced VEGF upregulation to adenosine, and adenosine receptors mediate the increase in $[Ca^{2+}]i$ levels and VEGF production in cultured DPC. SUR is proposed to be a target receptor for minoxidil and a source of ATP and adenosine production, respectively.

We postulated that the adenosine/VEGF pathway in cultured DPC might play an important role in hair growth. Our study was performed in cultured cells, however, which might be a limitation of this study. A recent *in situ* study (Yano *et al*, 2001) showed that upregulation of VEGF mRNA was found only in the outer root sheath keratinocytes, but not in DPC, of the anagen murine hair follicle. The angiogenic activity of the rat vibrissa hair follicle associated with the epithelial hair bulb (Stenn *et al*, 1988) might be partly due to this upregulation. A study by Lachgar *et al* (1998) and this study, however, showed definitively increased VEGF expression in cultured DPC by treatment with minoxidil. In addition, Kozlowska *et al* (1998) showed the immunohistochemical expression of VEGF in DPC. The discrepancy between *in vitro* and *in vivo* results has also been observed in the apoptosis of DPC. It is notable that DPC undergo apoptosis in culture (Ferraris *et al*, 1997) but appear not to during embryogenesis (Wessels and Roessner, 1965) and throughout the hair cycle in adults (Couchman, 1993; Nutbrown and Randall, 1995), suggesting that the normal quiescent state of DPC *in vivo* may confer protection from induction of cell death. As natural DPC exist in a complex epithelial–mesenchymal microenvironment, some characteristics *in vitro* may be suppressed in order to keep the physiologic balance. The inherent abilities of DPC might be released *in vivo* under the influence of special endogenous pathologic factors or exogenous stimulation. Given that adenosine-induced upregulation of VEGF expression is found in various cell types both *in vivo* (Martin *et al*, 1998) and *in vitro* (Gu *et al*, 1999), a similar mechanism on cultured DPC by minoxidil is expected to found *in vivo* under the influence of stimulation with topical minoxidil.

Herein, we propose a mechanism for minoxidil-induced VEGF production in cultured DPC, and conclude that an adenosine-mediated signal transduction pathway contributes to minoxidil-induced hair growth. The gene expression of a number of receptors such as SUR2B, along with that of adenosine receptors in DPC, emphasizes the important role of DPC in hair growth.

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REFERENCES

Aguilar-Bryan L, Clement JPIV, Gonzalez G, Kunjilwar K, Babenko A, Bryan J: Toward understanding the assembly and structure of KATP channels. *Physiol Rev* 78:227–245, 1998

Andersson KE: Clinical pharmacology of potassium channel openers. *Pharmacol Toxicol* 70:244–254, 1992

Awqati QA: Regulation of ion channels by ABC transporters that secrete ATP. *Science* 269:805–806, 1995

Buhl AE, Conrad SJ, Waldon DJ, Brunden MN: Potassium channel conductance as a control mechanism in hair follicles. *J Invest Dermatol* 101:148S–152S, 1993

Burton JL, Marshall A: Hypertrichosis due to minoxidil. *Br J Dermatol* 101:593–595, 1979

Burton JL, Schutt WH, Caldwell IW: Hypertrichosis due to diazoxide. *Br J Dermatol* 93:707–711, 1975

Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* 162:156–159, 1987

Couchman JR: Hair proteoglycans. *J Invest Dermatol* 101:60s–64s, 1993

Dargie HJ, Dollery CT, Daniel J: Minoxidil in resistant hypertension. *Lancet* 10: 515–518, 1977

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

Ferraris C, Cooklis M, Polakowska RR, Haake AR: Induction of apoptosis through the PKC pathway in cultured dermal papilla fibroblasts. *Exp Cell Res* 234:37–46, 1997

Goldberg MR: Clinical pharmacology of pinacidil, a prototype for drugs that affect potassium channels. *J Cardidiovasc Pharmacol* 12:S41–S47, 1988

Gu JW, Brady AL, Anand V, Moore MC, Kelly WC, Adair TH: Adenosine upregulates VEGF expression in cultured myocardial vascular smooth muscle cells. *Am J Physiol* 277:H595–H602, 1999

Hamaoka H, Minakuchi K, Miyoshi H, Arase S, Chen CH, Nakaya Y: Effect of K+ channel openers on K+ channel in cultured human dermal papilla cells. *J Med Invest* 44:73–77, 1997

Hashimoto E, Kage K, Ogita T, Nakaoka T, Matsuoka R, Kira Y: Adenosine as an endogenous mediator of hypoxia for induction of vascular endothelial growth factor mRNA in U-937 cells. *Biochem Biophys Res Commun* 204:318–324, 1994

Kato R, Ross S, Foex P: Fentanyl protects the heart against ischaemic injury via opioid receptors, adenosine A1 receptors and KATP channel linked mechanisms in rats. *Br J Anaesth* 84:204–214, 2000

Kirsch GE, Codina J, Birnbaumer L, Brown AM: Coupling of ATP-sensitive K+ channels to A1 receptors by G proteins in rat ventricular myocytes. *Am J Physiol* 259:H820–H826, 1990

Kitakaze M, Minamino T, Node K, *et al*: Role of activation of ectosolic 5′-nucleotidase in the cardioprotection mediated by opening of K+ channels. *Am J Physiol* 270:H1744–H1756, 1996

Kozlowska U, Blume-Peytavi U, Kodelja V, *et al*: Expression of vascular endothelial growth factor (VEGF) in various compartments of the human hair follicle. *Arch Dermatol Res* 290:661–668, 1998

Lachgar S, Moukadiri H, Jonca F, *et al*: Vascular endothelial growth factor is an autocrine growth factor for hair dermal papilla cells. *J Invest Dermatol* 106:17–23, 1996

Lachgar S, Charveron M, Gall Y, Bonafe JL: Minoxidil upregulates the expression of vascular endothelial growth factor in human hair dermal papilla cells. *Br J Dermatol* 138:407–411, 1998

Martin C, Yu AY, Jiang BH, Davis L, Kimberly D, Hohimer AR, Semenza GL: Cardiac hypertrophy in chronically anemic fetal sheep: increased vascularization is associated with increased myocardial expression of vascular endothelial growth factor and hypoxia-inducible factor 1. *Am J Obstet Gynecol* 178:527–534, 1998

Messenger AG: The culture of dermal papilla cells from human hair follicles. *Br J Dermatol* 110:685–689, 1984

Mukhopadhyay D, Akbarali HI: Depletion of $[Ca^{2+}]i$ inhibits hypoxia-induced vascular permeability factor (vascular endothelial growth factor) gene expression. *Biochem Biophys Res Commun* 229:733–738, 1996

Nakaya Y, Hamaoka H, Kato S, Arase S: Effect of minoxidil and pinacidil on single potassium channel current in cultured human outer root sheath cells and dermal papilla cells. *J Dermatol Sci* 7:S104–S108, 1994

Newgreen DT, Bray KM, McHarg AD, *et al*: The action of diazoxide and minoxidil on rat blood vessels: a comparison with cromakalim. *Br J Pharm* 100:605–613, 1990

Nutbrown M, Randall V: Differences between connective tissue–epithelial junctions in human skin and the anagen hair follicle. *J Invest Dermatol* 104:90–94, 1995

Olah ME, Stiles GL: The role of receptor structure in determining adenosine receptor activity. *Pharmacol Ther* 85:55–75, 2000

Rappolee DA, Mark D, Banda MJ, Werb Z: Wound macrophages express TGF-alpha and other growth factors *in vivo*: analysis by mRNA phenotyping. *Science* 241:708–712, 1988

Roscoe AK, Christensen JD, Lynch C III: Isoflurane, but not halothane, induces protection of human myocardium via adenosine A1 receptors and adenosine triphosphate-sensitive potassium channels. *Anesthesiology* 92:1692–1701, 2000

Schwanstecher M, Sieverding C, Dorschner H, Gross I, Aguilar-Bryan L, Schwanstecher C, Bryan J: Potassium channel openers require ATP to bind to and act through sulfonylurea receptors. *EMBO J* 17:5529–5535, 1998

Shryock JC, Belardinelli L: Adenosine and adenosine receptors in the cardiovascular system: biochemistry, physiology, and pharmacology. *Am J Cardiol* 79:2–10, 1997

Stenn KS, Fernandez LA, Tirrell SJ: The angiogenic properties of the rat vibrissa hair follicle associate with the bulb. *J Invest Dermatol* 90:409–411, 1988

Takagi H, King GL, Robinson GS, Ferrara N, Aiello LP: Adenosine mediates hypoxic induction of vascular endothelial growth factor in retinal pericytes and endothelial cells. *Invest Ophthalmol Vis Sci* 37:2165–2176, 1996

Toescu EC: Temporal and spatial heterogeneities of Ca^{2+} signaling: mechanisms and physiological roles. *Am J Physiol* 269:G173–G185, 1995

Wessels NK, Roessner KD: Nonproliferation in dermal condensations of mouse vibrissae and pelage hairs. *Dev Biol* 12:419–433, 1965

Yano K, Brown LF, Detmar M: Control of hair growth and follicle size by VEGF-mediated angiogenesis. *J Clin Invest* 107:409–417, 2001

Yokoshiki H, Sunagawa M, Seki T, Sperelakis N: ATP-sensitive K+ channels in pancreatic, cardiac, and vascular smooth muscle cells. *Am J Physiol* 274:C25–C37, 1998