British Journal of Pharmacology (1996) 118, 783–789 © 1996 Stockton Press All rights reserved 0007–1188/96 $12.00

Adenosine inhibitory effect on enhanced growth of aortic smooth muscle cells from streptozotocin-induced diabetic rats

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1 There is evidence to suggest that adenosine may regulate arterial smooth muscle cell (SMC) growth and proliferation, which is a key event in atherogenesis. This regulation may be mediated via adenylate cyclase. As diabetes is a known risk factor for atherosclerosis, we investigated the growth of aortic SMC from diabetic rats in primary culture and their sensitivity to adenosine and to adenylate cyclase activity.

2 Diabetes was induced with streptozotocin (STZ, 66 mg kg^{-1}, i.p.) Aortic SMC primary cultures were prepared from STZ-diabetic and age-matched rats 5 weeks after the STZ injection.

3 SMC from STZ-diabetic rats grew faster and reached greater densities at confluence than those from non-diabetic animals.

4 Adenosine inhibited growth in both control and diabetic SMC. However, cells from STZ-diabetic rats were apparently more sensitive to adenosine.

5 Direct activation of adenylate cyclase by forskolin induced a dose-dependent growth inhibition, similar in both groups of cells.

6 Cholera toxin, an activator of stimulatory GTP-binding protein (G_s), induced a similar growth inhibitory response in non-diabetic and diabetic SMC. Pertussis toxin (PTX), an inactivator of inhibitory GTP-binding protein (G_i), did not itself affect SMC growth. However, PTX increased dose-dependently the growth inhibition induced by adenosine in SMC from non-diabetic rats but not in SMC from diabetic rats.

7 These findings suggest a functional abnormality in G_i activity in SMC from diabetic rats, that would explain the increased sensitivity to the nucleoside. This impaired inhibitory pathway may reflect changes in the growth regulation of SMC in experimental diabetic states.

Keywords: Diabetes; atherosclerosis; smooth muscle cells; primary culture; adenosine

Introduction

The control of cellular proliferation is a fundamental biological process that has considerable practical importance in relation to human vascular diseases. In particular, the pathogenesis of both atherosclerosis and hypertension involves abnormal proliferation of smooth muscle cells (SMC) within the arterial wall (Ross, 1993). Diabetes mellitus is frequently associated with both diseases. However, little is known about the mechanisms which may connect diabetes to vascular complications. Hyperglycaemia and hyperinsulinaemia in addition to abnormalities of lipid metabolism may cause vascular dysfunctions (Ruderman & Haudennsschild, 1984; Colwell & Lopes-Virella, 1988). However, there is no conclusive evidence that diabetes mellitus contributes to progression of the atherosclerotic process by augmenting SMC proliferation. Recently, it has been reported that aortic SMC from streptozotocin-induced diabetic rats or alloxan-induced diabetic rabbits in explant culture grow faster than SMC from controls in the presence of serum or platelet-derived growth factor (Kawano *et al.*, 1993). However, SMC obtained from explant culture are only a selected subpopulation of the cells in each explant. Alternatively, enzyme-dispersed SMC in primary culture is another method of growing SMC, where, according to Campbell & Campbell (1993), the cells are more representative of those in the intact blood vessel.

Intimal SMC proliferation is thought to result from both the action of several mitogens (Thyberg *et al.*, 1990) and the diminution of inhibitory mediators (Newby *et al.*, 1992). Such inhibitors may include the endogenous vasodilator agents, prostacyclin (Owen, 1985) and nitric oxide (Garg & Hassid, 1989). However, SMC proliferation *in vivo* may also be a consequence of the responses of vascular smooth muscle to both inhibitory and stimulatory factors. Adenosine is one such vasodilator factor that is able to regulate cell proliferation. It may either inhibit or stimulate this process. Jonzon *et al.* (1985) reported that SMC proliferation could be either inhibited via an A_2 purinoceptor or stimulated via an A_1 subtype. The former seemed to involve an increase, and the latter a decrease, in adenosine 3′,5′-cyclic monophosphate (cyclic-AMP) levels. Furthermore, these authors showed an inverse relationship between cyclic AMP levels and DNA synthesis. However, little information is available on adenosine effects in SMC growth in diabetes.

Several groups have noted changes of adenosine sensitivity in experimental diabetes. Downing (1985) demonstrated a marked reduction in the coronary dilator activity of adenosine. In pancreas from experimental diabetic rats, adenosine was ineffective in stimulating glucagon secretion and its vasodilator effect was also reduced (Gross *et al.*, 1989). Similarly adipocytes from diabetic animals showed a reduction in the lipolysis inhibitory effect of adenosine (Green & Johnson, 1991). On the other hand, hippocampus from diabetic animals have an increased sensitivity to adenosine (Morrison *et al.*, 1992). Therefore, any change in the function of adenosine receptors could result in different effects. Adenosine receptors belong to the great family of G-protein linked receptors (Linden *et al.*, 1991). Interestingly, in the diabetic state G-protein alterations have been described, particularly in hepatocytes (Gawler *et al.*, 1987; Lynch *et al.*, 1989; Bushfield *et al.*, 1990), adipocytes (Strassheim *et al.*, 1990; Green & Johnson, 1991) and cardiac myocytes (Cros *et al.*, 1986).

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To investigate the growth of vascular SMC in diabetes, we studied the growth of aortic SMC from streptozotocin-diabetic rats in primary cultures. We also evaluated the response to adenosine and the functional activity of the GTP-binding proteins in aortic SMC from streptozotocin-diabetic rats.

Methods

Induction of diabetes

Adult male Wistar rats (Iffa Credo, l'Arbresle, France), weighing 320–350 g, were used in all experiments. They were randomly distributed and housed individually during a 10 day acclimatization period with laboratory chow and water *ad libitum*. Diabetes was induced with a single i.p. injection of streptozotocin (STZ, Upjohn Co., Kalamazoo, Mich, U.S.A.) (66 mg kg^{-1}, dissolved in citrate buffer). The diabetic state was then ensured by daily measurements of glucosuria. Animals with clear glucosuria (higher than 55 mmol per 24 h) after STZ-injection were deemed diabetic. Control animals were injected with an equivalent volume of vehicle. Five weeks after induction of diabetes, the rats were killed by cervical dislocation and intracardiac blood samples were immediately collected. The thoracic aortae were harvested for SMC preparation.

Biochemical assays

Blood glucose and glucosuria were measured by the potassium ferricyanide method with a Technicon autoanalyser (Alric *et al.*, 1965). Insulin concentration was determined according to the method of Herbert *et al.* (1965). Rat insulin was used as a standard. The anti-insulin serum was supplied by Miles Laboratories (Paris). The sensitivity of the method was 13.2 pM. Glucagon concentration was measured according to the radioimmunological method described by Unger *et al.* (1970). We used the BR 124 antiglucagon serum supplied by the Institut de Biochimie Clinique of Geneva. The sensitivity of the method was 15 ng l^{-1}. Results were given as nanogram equivalents of porcine glucagon.

Cell preparation and culture

SMC were isolated from control and STZ-diabetic rat aortae by enzymatic dissociation as described by Bodin *et al.* (1991). Aortae were removed aseptically and placed in culture medium composed of equal parts of Eagle's minimum essential medium (MEM) and Ham's F-10 with Earle's salts (both containing a normal glucose concentration of 5.5 mM, Eurobio, France), supplemented with 2 mM glutamine, 0.05 mM vitamin C, 0.05 mM L-proline, 100 u ml^{-1} penicillin G and 2% (vol/vol) serum substitutes Ultroser SF/Ultroser G (3:1 vol/vol, Sepracor SA., Villeneuve la Garenne, France). Aortae were incubated for 20 min at 37°C in Hank's balanced salt solution (HBSS, Eurobio, France) containing 120 u ml^{-1} collagenase (type II, Worthingtonn, Biochemical Corp., Freehold, NJ, U.S.A.). Aortae were placed in complete culture medium and the adventicia and endothelium were gently removed. Media were further incubated for 60 min at 37°C in HBSS containing both collagenase (150 u ml^{-1}) and elastase (100 u ml^{-1}) (Boehringer Mannheim France S.A.). The digested tissues were gently pipetted up and down 10 times through a 10 ml sterile pipette and sieved through a 180 μm mesh. The resulting cell suspension was diluted 20 times with complete culture medium and centrifuged at 200 g for 7 min at room temperature. The SMC from control and STZ-diabetic rats were resuspended in culture medium, counted with a Thoma haemocytometer and seeded into 24-well plates (Corning-Col) at 2×10^4 cm^{-2}. The culture plates were incubated in a humidified incubator at 37°C in 5% CO_2 and 95% room air.

Cell growth

To compare SMC growth from normal and STZ-diabetic rats in primary culture, cells were plated at the same concentrations and maintained in normal culture medium until they were postconfluent (10–15 days postculture). The growth of SMC was determined twice a week.

To study the effects of adenosine on cell growth, cultures were initiated in complete culture medium (day 0) in the presence or absence of increasing adenosine concentrations. The growth of SMC was evaluated after 3, 6. 10 and 13 days of incubation. To study the effects of adenylate cyclase activation on SMC growth, cells were incubated from day 0 in the absence or presence of increasing forskolin concentrations. Cell growth was evaluated after the 15-d incubation period.

To evaluate G-protein function in SMC growth, cells were incubated with or without cholera (1 μg ml^{-1}) or pertussis (0.1 μg ml^{-1}) toxins over a 15-d-period. To evaluate Gi-protein function in the effect of adenosine on SMC growth, cells were incubated with or without adenosine (1 to 10 μM), in the presence or absence of pertussis toxin (0.1 μg ml^{-1}). Cell growth was determined after the 15-d incubation period.

In all experiments, final test agent concentrations were obtained in each well with a test solution volume 10× concentrated. The culture medium was changed for fresh medium with or without fresh test agent solutions two times a week.

The net growth of SMC in culture was evaluated by the dye uptake method (Vilcek *et al.*, 1986). At intervals, medium was removed and cells were washed with Dulbecco's phosphate buffered saline (PBS) and fixed with 10% formalin in 9% acetic acid/0.1 M sodium acetate buffer for 15 min. Then, cells were stained with a 0.5% solution of the protein-binding dye, amido black 10B (naphtol blue black, Sigma Chemical Co.) in 9% acetic acid with 0.1 M sodium acetate for 25–30 min. After three washes in distilled water to remove unbound dye, the bound dye was eluted by sodium hydroxide (50 mM). Absorbance of each well was read at 630 nm.

Drugs

Adenosine, forskolin and cholera toxin were purchased from Sigma Chemical Co (St. Louis, Mo, U.S.A.). Pertussis toxin was obtained from List Biological Laboratories (Campbell, CA, U.S.A.). Adenosine was directly dissolved in complete culture medium. Forskolin and both bacterial toxins were dissolved in ethanol and sterile water, respectively, before dilution to the required concentrations in the culture medium. The amount of ethanol in the final incubation medium did not exceed 0.1%. When used alone at this concentration, the solvent did not modify SMC growth (data not shown).

Data analysis

Data are expressed as mean ± s.e.mean. Dose-response curves were constructed by expressing proliferation in absolute values or in some cases as a percentage of the maximal response to adenosine. Each dose-response curve was characterized by determining the EC_{50} value (the dose of the drug inducing a half-maximal response), and by determining the maximal response to the drug. Differences between two means were determined using the Student's unpaired t test; $P<0.05$ was considered statistically significant.

Results

Streptozotocin-induced diabetic state

On the day rats were killed, STZ-treated animals were effectively diabetic (Table 1). Plasma glucose concentrations were four times greater than controls. The animals were markedly glucosuric. Plasma insulin concentrations were drastically decreased, and plasma glucagon concentrations showed a two-

Table 1 Characteristics of rats five weeks after induction of diabetes with streptozotocin, just before aortae were removed

	n	*Body weight (g)*	*Urine glucose (mmol per 24 h)*	*Plasma glucose (mM)*	*Plasma insulin (pM)*	*Plasma glucagon (ng l^{-1})*	*Plasma cholesterol (mM)*	*Plasma triglycerides (mM)*
Control	8	471±8	0	9.6±0.3	483±52	144±7	0.96±0.16	1.01±0.17
Diabetic	10	309±10*	78.3±5.6*	37.6±2.7*	52±9*	297±15*	2.03±0.16*	2.50±0.50†

Values are means±s.e.mean. *$P<0.001$ and †$P<0.5$ versus control value.

fold increase. The mean body weight increase in age-matched control rats was completely suppressed in STZ-diabetic rats. Furthermore, the diabetic state induced by STZ was associated with significant elevations in plasma cholesterol and triglyceride concentrations.

Growth of aortic SMC from STZ-diabetic and non-diabetic rats

SMC from both STZ-diabetic and non-diabetic rats showed a similar pattern of growth curves in primary cultures (Figure 1). Cell growth started on day 3, rose continuously and confluence was achieved on days 10 to 13. However, values of SMC from STZ-diabetic rats were higher at all time points. Although cells were seeded at identical density, SMC growth from STZ-diabetic rats was significantly increased after 3 days in primary culture ($P<0.001$) and continued to be higher than that from non-diabetic rats. At confluence, values of SMC from STZ-diabetic rats were about 1.5 times greater than those of SMC from non-diabetic rats ($P<0.001$).

Effect of adenosine on the growth of aortic SMC from STZ-diabetic and non-diabetic rats

The effects of adenosine were measured after 3 days, i.e. at the start of cell outgrowth, after 6 days, i.e. in the exponential growth phase, and after 10 days, when cells reached a quiescent growth state.

After 6 and 10 days of culture, adenosine at 10 and 100 μM inhibited the net growth of both non-diabetic and STZ-diabetic SMC (Figure 2a and b). The highest concentration of adenosine completely abolished cell growth. This inhibitory effect was reversible, as determined by SMC growth in 10-d cultures after 4 days in the absence of adenosine (100 μM) (data not shown). 1 μM of adenosine induced growth inhibition (40.7±4.7%) only on STZ-diabetic SMC in 6-d cultures. No inhibitory effect at 1 μM was shown either on STZ-diabetic or on non-diabetic SMC in 10-d cultures. Furthermore, adenosine was able to inhibit the outgrowth of STZ-diabetic SMC as early as 3 days of exposure to either concentration tested, whereas there was no significant effect on the growth of non-diabetic SMC (Figure 3). When cells were confluent (10–13 days), comparison of two dose-effect curves (Figure 4) showed that the EC_{50} for adenosine was not significantly modified by the STZ-diabetic state (3.3 μM in non-diabetic SMC versus 2.7 μM in STZ-diabetic SMC).

Effect of forskolin

Adenosine receptor activation has been related to an inhibition (A_1 receptors) or to a stimulation (A_2 receptors) of cyclic AMP production. In order to determine which adenylate cyclase system is involved in the inhibitory effect of adenosine on SMC growth, forskolin, a direct stimulator of adenylate cyclase, was tested. Forskolin inhibited SMC growth in a concentration-dependent manner (Figure 5) in both groups. However, SMC from non-diabetic and STZ-diabetic rats did not differ significantly in their response to forskolin (the EC_{50} was 0.16 μM in control cell preparation and 0.13 μM in diabetic cell preparation).

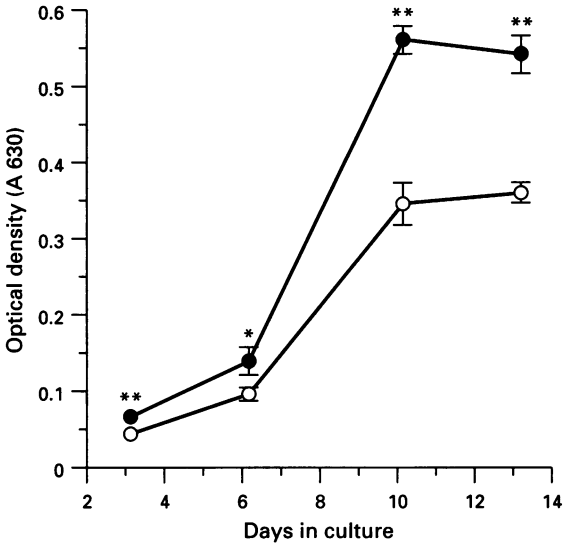

Figure 1 Growth of cultured aortic SMC from non-diabetic (○) and 5-wk streptozotocin-induced diabetic (●) rats. Values shown are means±s.e.mean of 10–12 wells from three experiments. *$P<0.05$, **$P<0.001$ compared with non-diabetic values.

Effect of bacterial toxins

As shown in Figure 6, activation of stimulatory GTP-binding protein (G_s) with cholera-toxin (CTX, 1 μg mg^{-1}) inhibited SMC growth identically both in non-diabetic (68.5±1.2%) and in STZ-diabetic SMC (64.5±1.6%). In contrast, inactivation of inhibitory GTP-binding protein (G_i) with pertussis-toxin (PTX, 0.1 μg ml^{-1}) did not *per se* affect SMC growth. Furthermore, PTX-treatment shifted to the left the concentration-dependent curve of adenosine in non-diabetic SMC (Figure 7a), without modification of the inhibitory effect at 10 μM adenosine. In contrast, PTX-treatment did not modify the adenosine effect in STZ-diabetic SMC (Figure 7b).

Discussion

Our results show that aortic SMC from STZ-diabetic rats exhibit enhanced growth in primary cultures and that they were more susceptible to the growth inhibitory effects of adenosine.

The outgrowth of STZ-diabetic SMC is in agreement with the observations in 2nd-passaged, explant-derived SMC cultures (Kawano *et al.*, 1993). The enhanced growth of SMC from STZ-induced diabetic rats was observed after 3 days of primary culture. It is known that changes from a contractile to a synthetic phenotype precede the onset of cellular proliferation (Thyberg *et al.*, 1990). Therefore, our results suggest that STZ-diabetic SMC undergo a more rapid change in phenotype than non-diabetic SMC or that they may have already undergone a phenotypic modulation *in vivo* in STZ-induced diabetic rats. Similar results have been observed using aortic SMC from different animal models of diabetes, such as the alloxan-induced diabetic rabbit (Kawano *et al.*, 1993) and the BioBreeding (BB) spontaneous autoimmune diabetic rat (Larson & Haudenschild, 1988), suggesting that the alterations

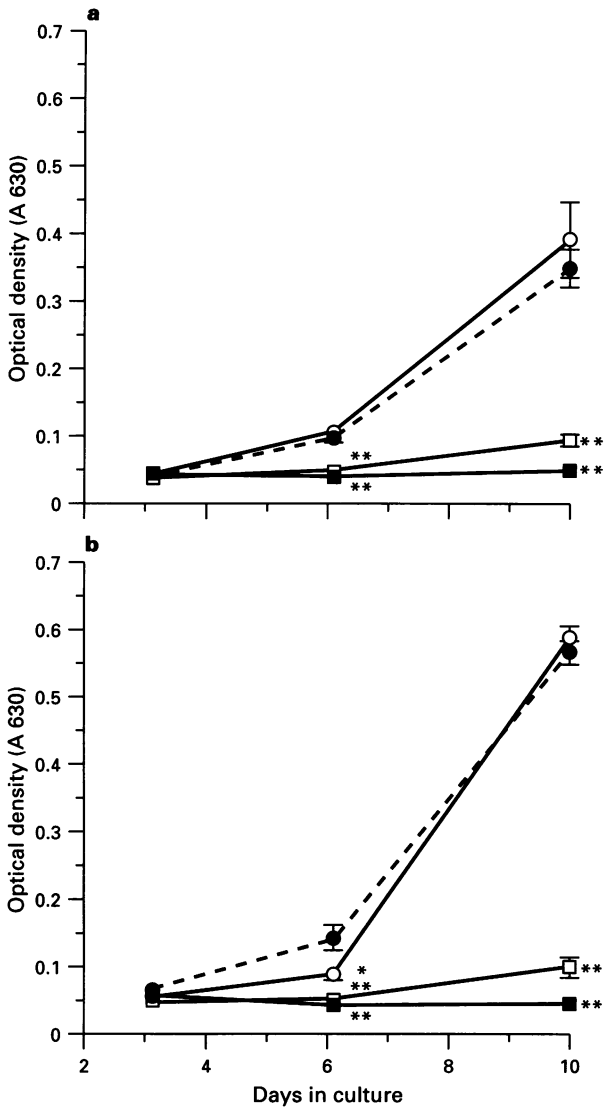

Figure 2 Growth curves of cultured aortic SMC from non-diabetic (a) and 5-wk streptozotocin-induced diabetic (b) rats in response to different adenosine concentrations: (●) control; (○) 1 μM; (□) 10 μM and (■) 100 μM adenosine. SMC were incubated from the beginning of the experiments in the presence or absence of adenosine indicated concentrations. Values shown are means ± s.e.mean of 10–18 wells from three experiments. $*P<0.01$, $**P<0.001$ compared with control values.

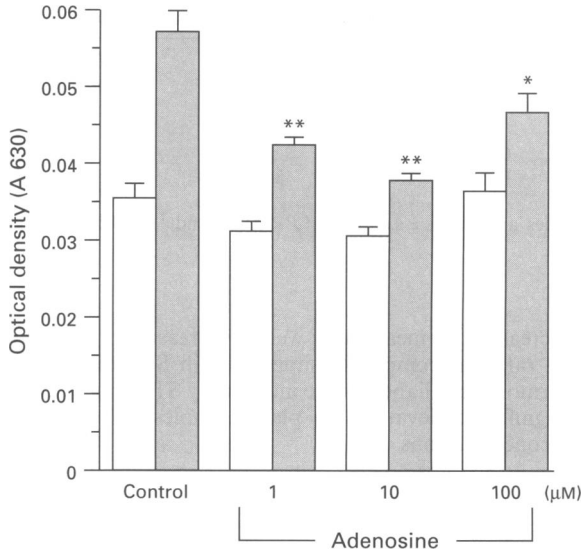

Figure 3 Effect of adenosine on the early growth of cultured aortic SMC from non-diabetic (open columns) and 5-wk streptozotocin-induced diabetic (shaded columns) rats. SMC were incubated from time 0 to 72 h in the presence or absence of adenosine at the indicated concentrations. Values shown are means ± s.e.mean of 12 wells from two experiments. $*P<0.05$, $**P<0.01$, $***P<0.001$ compared with control values.

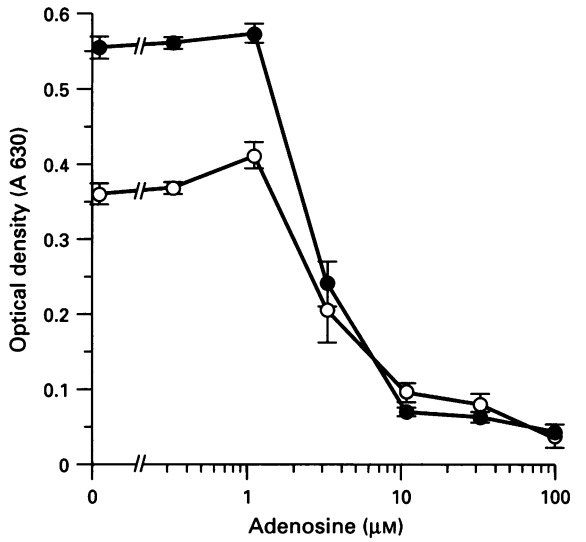

Figure 4 Dose-response curves for the growth effect of adenosine in cultured aortic SMC from non-diabetic (○) and 5-wk streptozotocin-induced diabetic (●) rats after the 10-day incubation. Values shown are means ± s.e.mean of 8–10 wells from two experiments.

of STZ-diabetic SMC in culture are not due to an effect of streptozotocin itself. Moreover, the presence of streptozotocin in the culture medium had no effect on cell growth (data not shown). Our STZ-induced diabetic animals were clearly hyperglycaemic, but Sakakibara *et al.* (1993) recently reported that elevated glucose concentrations had no effect on rabbit cultured SMC growth. Indeed, in our experimental conditions the outgrowth of normal SMC was not modified by the incubation with high glucose concentrations (data not shown). Non-diabetic animals being age-matched, we can rule out the influence of aging *per se* in the enhanced growth of STZ-diabetic SMC (Hariri *et al.*, 1988). Thus, the present *in vitro* findings may reflect the properties of SMC due to the experimental diabetic state itself.

SMC from STZ-diabetic rats showed increased sensitivity to the growth inhibitory effect of adenosine. The growth inhibition of experimental-diabetic SMC was observed after 3 days of culture, while no effect was observed in control SMC after this delay. This suggests that adenosine acts on cells immediately after transformation into the synthetic phenotype. This explanation is consistent with the inhibition of DNA synthesis that has been reported in preparations of synthetic SMC in response to adenosine (Jonzon *et al.*, 1985). One possible explanation for the supersensitivity to adenosine of diabetic SMC is that in the media *in vivo*, they might be converted to the synthetic phenotype, whereas normal SMC are known to retain the contractile phenotype (Campbell & Campbell, 1993). However, our results do not rule out an additional effect of adenosine on SMC in the contractile state. The inhibition as observed in the present study was reversible and not reproduced by inosine (data not shown), the first product of adenosine degradation. These results suggest that the effect of adenosine was unlikely to be mediated by cytotoxicity or metabolic inhibition (Mirkin & Slomiany, 1985).

In the growth phase, diabetic SMC showed an increased sensitivity to adenosine after 6 days of culture. These findings suggest that the difference in the response of normal and diabetic SMC to adenosine is probably due to different synthetic

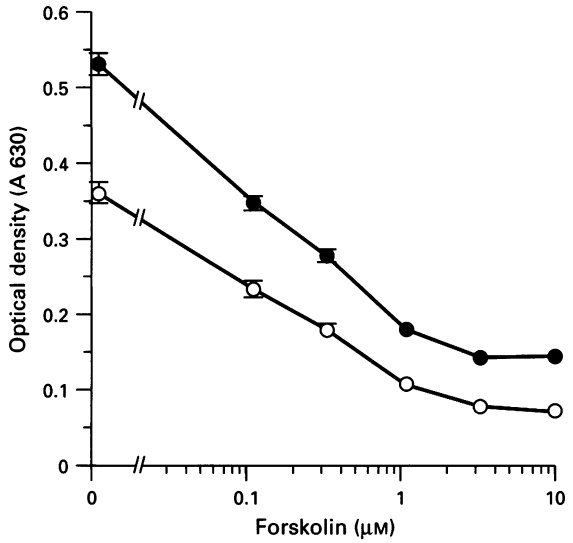

Figure 5 Dose-response curves for the growth effect of forskolin in cultured aortic SMC from non-diabetic (○) and 5-wk streptozotocin-induced diabetic (●) rats after the 15-day incubation. Values shown are means ± s.e.mean of 8 wells from two experiments.

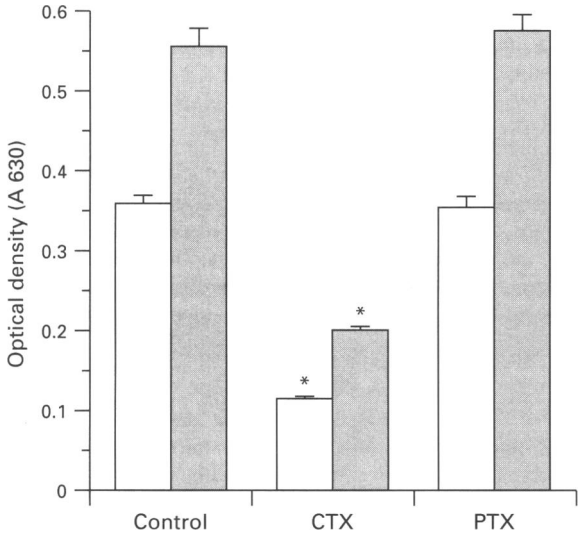

Figure 6 Effect of cholera toxin (CTX, 1 $\mu g\,ml^{-1}$) and pertussis toxin (PTX, 0.1 $\mu g\,ml^{-1}$) on the growth of cultured aortic SMC from non-diabetic (open columns) and 5-wk streptozotocin-induced diabetic (shaded columns) rats after the 15-day incubation. Values shown are means ± s.e.mean of 8 wells from two experiments. $^*P<0.001$ compared with control values.

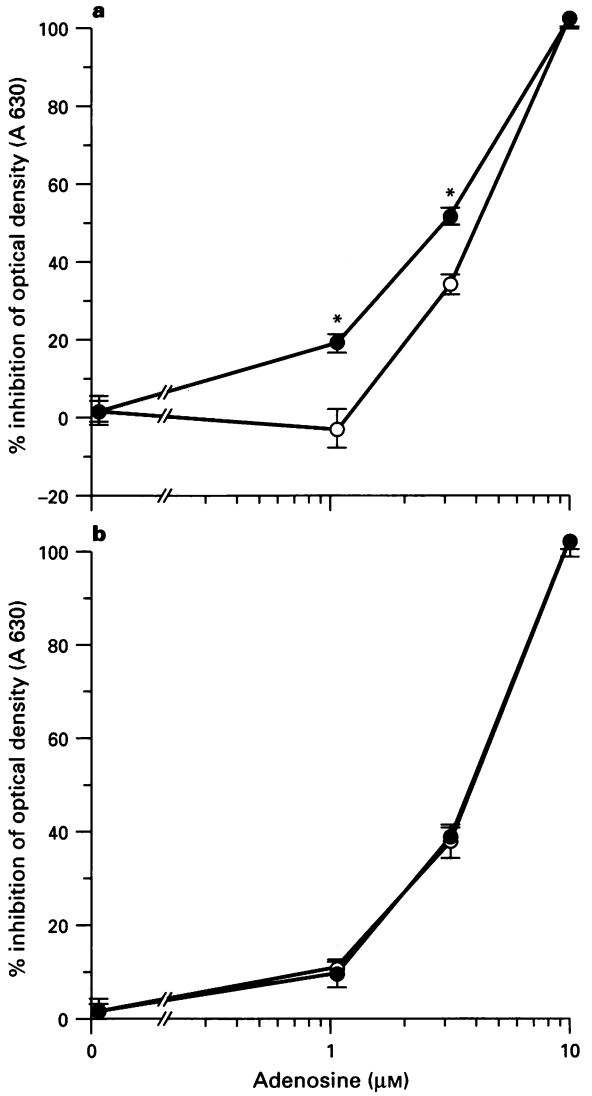

Figure 7 Effect of adenosine on the growth of cultured aortic SMC from non-diabetic (a) and 5-wk streptozotocin-induced diabetic (b) rats in the presence (●) or absence (○) of pertussis toxin (PTX, 0.1 $\mu g\,ml^{-1}$), after the 15-day incubation. Data are expressed as a percentage of maximal adenosine-induced growth inhibition (100%) assessed in the absence of PTX. Values shown are means ± s.e.mean of 8 wells from two experiments. $^*P<0.001$ compared with control values.

phenotypes. Our data differ from those obtained on coronary vessels (Downing, 1985), adipocytes (Green & Johnson, 1991) and pancreas (Gross *et al.*, 1989) in which a decreased sensitivity to this purine was observed. In all cases, the differences were attributed to a dysfunction in the response to the activation of adenosine receptors induced by the experimental diabetic state. In the adipocytes, the mechanism of the decreased antilipolytic sensitivity was attributed to a decrease in the A_1 receptor coupling to adenylate cyclase (Green & Johnson, 1991). On the other hand, the suppressed α-cell response in the isolated perfused pancreas of the rat and the reduction of the vasodilator effect in the pancreatic vascular bed was related to an impairment in the response to the A_2 adenosine receptor (Gross *et al.*, 1989). The adenosine-induced decrease in DNA synthesis was attributed to the adenylate cyclase activation via the A_2 receptors (Jonzon *et al.*, 1985). The present work indicates not only a preserved adenosine receptor response but an increase of adenosine sensitivity in cultured aortic SMC from STZ-diabetic rats. However, it can be observed that the work quoted above shows functional alterations in different preparations (tissue, organ or cell type) from STZ-diabetic rats, whereas the present work shows alterations in the growth of SMC from STZ-diabetic rats, a function observed only after cell phenotype modulation to a synthetic state.

To investigate the mechanism of the increased sensitivity of STZ-diabetic SMC to adenosine, we studied the functional activity of adenylate cyclase. Adenosine is believed to inhibit growth by binding to A_2 receptors and stimulating adenylate cyclase via G regulatory proteins. Several groups suggested the possibility that the adenylate cyclase system is altered in diabetes. However, the results of these studies have been conflicting. To study the catalytic activity of the enzyme in STZ-diabetic rats, forskolin-stimulated adenylate cyclase activity has been reported to be unchanged in cardiac myocytes (Nishio *et al.*, 1988) and adipocyte membranes (Strassheim *et al.*, 1990), and either unchanged (Lynch *et al.*, 1989) or decreased (Gawler *et al.*, 1987) in liver plasma membranes. We found that forskolin, a direct activator of this enzyme, inhibited SMC growth, although responses were similar in STZ-

diabetic and non-diabetic SMC. Since the forskolin responses were not altered in SMC from STZ-diabetic rats, the increased responses of SMC growth to adenosine might be due to changes occurring before the activation of adenylate cyclase. This explanation was supported by the fact that stimulation of adenylate cyclase activity by isoprenaline was enhanced in adipocyte membranes from STZ-diabetic rats in comparison with that found in membranes from non-diabetic controls (Strassheim *et al.*, 1990). Furthermore, this finding supports a link between the adenosine inhibitory effect to an increase of adenylate cyclase activity probably via an A_2 purinoceptor subtype, as previously reported by Jonzon *et al.* (1985).

It seems likely that the increased sensitivity to adenosine of SMC from STZ-diabetic rats could result from a change at the level of GTP-binding proteins (G_s and G_i proteins). Gawler *et al.* (1987) reported that G_i is almost absent in livers from STZ-diabetic rats. However, more recently it has been reported that there are normal amounts of G_i but increased levels of G_s in livers from STZ-diabetic rats (Lynch *et al.*, 1989). In the adipocyte membranes, it has been suggested that diabetes causes selective changes in the functioning of G_i (Strassheim *et al.*, 1990; Green & Johnson, 1991). Thus, it is possible that this molecule is ADP ribosylated in diabetes (Green & Johnson, 1991). We found no differences between non-diabetic and STZ-diabetic SMC in response to cholera toxin or pertussis toxin. However, adenosine, in the presence of pertussis toxin, had a greater growth inhibitory effect in SMC from non-diabetic rats than in SMC from STZ-diabetic rats. An enhanced stimulatory effect has also been observed for both isoprenaline- (Katada *et al.*, 1982) and glucagon- (Heyworth *et al.*, 1984) mediated stimulation of adenylate cyclase activity in C6 glioma cell and hepatocyte membranes, respectively, from normal animals whose cells were pretreated with pertussis toxin in order to inactivate G_i. Our findings suggest that there is less functional G_i in SMC from STZ-diabetic rats. Thus, inactivation of G_i in diabetic states could remove a tonic inhibitory effect on receptor-G_s mediated activation of adenylate cyclase, which could lead to enhanced stimulatory receptor responses. This finding provides a mechanism for the increased adenosine sensitivity of SMC from STZ-diabetic rats.

The impairment in G_i function seems to conflict with the increase of SMC growth in experimental diabetes. If the tonic inhibitory effect upon adenylate cyclase was abolished in SMC from STZ-diabetic animals, this might lead to an elevation of adenylate cyclase activity in basal conditions. However, the enzyme is known to be a negative regulator of SMC proliferation (Jonzon *et al.*, 1985). One explanation for this discrepancy could be the fact that basal adenylate cyclase activity would itself be reduced as previously reported in both adipocytes (Strassheim *et al.*, 1990) and hepatocytes (Gawler *et al.*, 1987) from STZ-diabetic animals. Another possible explanation could be an enhanced response to serum mitogens such as the plateled-derived growth factor (Kawano *et al.*, 1993).

Our results support the role of adenosine as an inhibitory trophic factor of blood vessel structure. Indeed, *in vitro* findings show that adenosine receptor agonists inhibit DNA synthesis in cultured SMC (Jonzon *et al.*, 1985) and *in vivo* findings show that hypertrophic-hyperplastic changes in the vasculature after sympathetic denervation could be mediated by the loss of a repressive effect of adenosine on receptors of effector cells (Matias *et al.*, 1991). On the other hand, several arguments suggest that the local concentration of adenosine may be reduced in the diabetic state. First, the reduction in the plasma insulin concentration, as observed in STZ diabetes, leads to an increased activity of adenosine deaminase, the principal enzyme involved in the nucleoside degradation (Rutkiewicz & Górski, 1990). Furthermore, it was shown that the hypertriglyceridaemia formed in STZ-induced diabetes could be normalized by the activation of adenosine receptors *in vivo* (Hoffman *et al.*, 1986). Thus, SMC growth regulation by adenosine may be affected by the diabetic state.

In conclusion, we demonstrated that aortic SMC from STZ-diabetic rats show increased ability to grow in primary cultures. There was an increased sensitivity to the growth inhibitory effect of adenosine, apparently due to an impairment in G_i function. These findings may reflect defects in the growth regulation of SMC in experimental diabetic states and these characteristics may contribute to an increased development of smooth muscle cells in vascular alterations of diabetes.

We thank Dr Antoine Avignon for careful and critical review of the manuscript.

References

ALRIC, R., MARIANI, M.M. & LOUBATIÈRES, A.L. (1965). Importance de l'etat des éléments figurés du sang et en particulier de celui des globules rouges sur les valeurs du glucose sanguin mesuré par l'auto-analyseur Technicon. *Pathol. Biol.*, **13**, 506–511.

BODIN, P., RICHARD, S., TRAVO, C., BERTA, P., STOCLET, J.-C., PAPIN, S. & TRAVO, P. (1991). Responses of subcultured rat aortic smooth muscle myocytes to vasoactive agents and KCl-induced depolarization. *Am. J. Physiol.*, **260**, C151–C158.

BUSHFIELD, M., GRIFFITHS, S.L., MURPHY, G.J., PYNE, N.J., KNOWLER, J.T., MILLIGAN, G., PARKER, P.J., MOLLNER, S. & HOUSLAY, M.D. (1990). Diabetes-induced alterations in the expression, functioning and phosphorylation state of the inhibitory guanine nucleotide regulatory protein Gi-2 in hepatocytes. *Biochem. J.*, **271**, 365–372.

CAMPBELL, J.H. & CAMPBELL, G.R. (1993). Culture techniques and their applications to studies of vascular smooth muscle. *Clin. Sci.*, **85**, 501–513.

COLWELL, J.A. & LOPES-VIRELLA, M.F. (1988). A review of the development of large vessel disease in diabetes mellitus. *Am. J. Med*, **85** (Supp. 5A), 113–118.

CROS, G.H., CHANEZ, P.O., MICHEL, A., MCNEILL, J.H. & SERRANO, J.J, (1986). Cardiac β-adrenergic receptors in diabetic rats: alteration of guanyl nucleotide regulation. *J. Pharmacol.*, **17**, 595–600.

DOWNING, S.E. (1985). Restoration of coronary dilator action of adenosine in experimental diabetes. *Am. J. Physiol.*, **249**, H102–H107.

GARG, U.C. & HASSID, A. (1989). Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J. Clin. Invest.*, **83**, 1774–1777.

GAWLER, D., MILLIGAN, G., SPIEGEL, A.M., UNSON, C.G. & HOUSLAY, M.D. (1987). Abolition of the expression of inhibitory guanine nucleotide regulatory protein Gi activity in diabetes. *Nature*, **327**, 229–232.

GREEN, A. & JOHNSON, J.L. (1991). Evidence for impaired coupling of receptors to Gi protein in adipocytes from streptozocin-induced diabetic rats. *Diabetes*, **40**, 88–94.

GROSS, R., HILLAIRE-BUYS, D., BERTRAND, G., RIBES, G. & LOUBATIÈRES-MARIANI, M.M. (1989). Diabetes and impaired response of glucagon cells and vascular bed to adenosine in rat pancreas. *Diabetes*, **38**, 1291–1295.

HARIRI, R.J., HAJJAR, D.P., COLETTI, D., ALONSO, D.R., WEKSLER, M.E. & RABELLINO, E. (1988). Aging and arteriosclerosis. Cell cycle kinetics of young and old arterial smooth muscle cells. *Am. J. Pathol.*, **131**, 132–136.

HERBERT, V., LAW, K.S., GOTLIEB, C.W. & BLEICHER, S.J. (1965). Coated charcoal immunoassay of insulin. *J. Clin. Endocrinol. Metab.*, **25**, 1375–1384.

HEYWORTH, C.M., HANSKI, E. & HOUSLAY, M.D. (1984). Islet activating protein blocks glucagon desensitization in intact hepatocytes. *Biochem. J.*, **222**, 189–194.

HOFFMAN, B.B., DALL'AGLIO, E., HOLLENBECK, C., CHANG, H. & REAVEN, G.M. (1986). Suppression of free fatty acids and triglycerides in normal and hypertriglyceridemic rats by the adenosine receptor agonist phenylisopropyladenosine. *J. Pharmacol. Exp. Ther.*, **239,** 715–718.

JONZON, B., NILSSON, J. & FREDHOLM, B.B. (1985). Adenosine receptor-mediated changes in cyclic AMP production and DNA synthesis in cultured arterial smooth muscle cells. *J. Cell. Physiol.*, **124,** 451–456.

KATADA, T., AMANO, T. & UI, M. (1982). Modulation by islet-activating protein of adenylate cyclase activity in C6 glioma cells. *J. Biol. Chem.*, **257,** 3739–3746.

KAWANO, M., KOSHIKAWA, T., KANZAKI, T., MORISAKI, N., SAITO, Y. & YOSHIDA, S. (1993). Diabetes mellitus induces accelerated growth of aortic smooth muscle cells: association with overexpression of PDGF β-receptors. *Eur. J. Clin. Invest.*, **23,** 84–90.

LARSON, D.M. & HAUDENSCHILD, C.C. (1988). Activation of smooth muscle cell outgrowth from BB/Wor rat aortas. *Diabetes*, **37,** 1380–1385.

LINDEN, J., TUCKER, A.L. & LYNCH, K.R. (1991). Molecular cloning of adenosine A1 and A2 receptors. *Trends Pharmacol. Sci.*, **10,** 326–328.

LYNCH, C.J., BLACKMORE, P.F., JOHSON, E.H., WANGE, R.L., KRONE, P.K. & EXTON, J.H. (1989). Guanine nucleotide binding regulatory proteins and adenylate cyclase in livers of streptozotocin- and BB/Wor-diabetic rats: immunodetection of Gs and Gi with antisera prepared against synthetic peptides. *J. Clin. Invest.*, **83,** 2050–2062.

MATIAS, A., ALBINO-TEIXEIRA, A., POLÓNIA, J. & AZEVEDO, I. (1991). Long-term administration of 1,3-dipropyl-8-sulfophenylxanthine causes arterial hypertension. *Eur. J. Pharmacol.*, **193,** 101–104.

MIRKIN, B.L. & SLOMIANY, D.J. (1995). Effects of purines on cell growth and differentiation. In *Purines. Pharmacology and Physiological Roles*. ed. Stone, T.W. pp. 233–243. London: Macmillan Press.

MORRISON, P.D., MACKINNON, M.W.B., BARTRUP, J.T., SKETT, P.G. & STONE, T.W. (1992). Changes in adenosine sensitivity in the hippocampus of rats with streptozotocin-induced diabetes. *Br. J. Pharmacol.*, **105,** 1004–1008.

NEWBY, A.C., SOUTHGATE, K.M. & ASSENDER, J.W. (1992). Inhibition of vascular smooth muscle cells proliferation by endothelium-dependent vasodilators. *Herz*, **17,** 291–299.

NISHIO Y, KASHIWAGI, A., KIDA, Y., KODAMA, M., ABE, N., SAEKI, Y & SHIGETA, Y. (1988). Deficiency of cardiac β-adrenergic receptor in streptozotocin-induced diabetic rats. *Diabetes*, **37,** 1181–1187.

OWEN, N.E. (1985). Prostacyclin can inhibit DNA synthesis in vascular smooth muscle cells. In *Prostaglandins, Leukotrienes, and Lipoxins*. ed. Bailey, J.M. pp. 193–204. New York: Plenum Publishing Corporation.

ROSS, R. (1993). The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature*, **362,** 801–809.

RUDERMAN, N.B. & HAUDENNSSCHILD, C. (1984). Diabetes as an atherogenic factor. *Prog. Cardiovasc. Dis.*, **26,** 373–412.

RUTKIEWICZ, J. & GÓRSKI, J. (1990). On the role of insulin in regulation of adenosine deaminase activity in rat tissues. *FEBS Lett.*, **271,** 79–80.

SAKAKIBARA, F., HOTTA, N., KOH, N. & SAKAMOTO, N. (1993). Effects of high glucose concentrations and Epalrestat on sorbitol and myo-Inositol metabolism in cultured rabbit aortic smooth muscle cells. *Diabetes*, **42,** 1594–1600.

STRASSHEIM, D., MILLIGAN, G. & HOUSLAY, M.D. (1990). Diabetes abolishes the GTP-dependent, but not the receptor-dependent inhibitory function of the inhibitory guanine-nucleotide-binding regulatory protein (Gi) on adipocyte adenylate cyclase activity. *Biochem. J.*, **266,** 521–526.

THYBERG, J., HEDIN, U., SJÖLUND, M., PALMBERG, L. & BOTTGER, B.A. (1990). Regulation of differentiated properties and proliferation of arterial smooth muscle cells. *Arteriosclerosis*, **10,** 966–990.

UNGER, R.H., AGUILAR-PARADA, E., MÜLLER, W. & EISENTRAUT, A.M. (1970). Studies of pancreatic alpha cell function in normal and diabetic subjects. *J. Clin. Invest.*, **48,** 837–848.

VILCEK, J., PALOMBELLA, V.J., HENRIKSENN-DESTEFANO, D., SWENSON, C., FEINMAN, R., HIRAI, M. & TSUJIMOTO, M. (1986). Fibroblast growth enhancing activity of tumor necrosis factor and its relationship to other polypeptide growth factors. *J. Exp. Med.*, **163,** 632–643.

(Received September 20, 1995
Revised February 1, 1996
Accepted February 15, 1996)