Differential Inhibitory Effects of Antidiabetic Drugs on Arterial Smooth Muscle Cell Proliferation

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We compared three drugs representing different classes of antidiabetic pharmacology (glyburide, a sulfonylurea; pioglitazone, a thiazolidinedione; and metformin, a biguanide) in terms of their direct effects on proliferation of cultured arterial smooth muscle cells (SMC). Rat aortic SMC were seeded at $4 \times 10^4$ cells per well. After 24 h, they were treated every 2 to 4 days for 2 weeks with 5% fetal bovine serum (FBS) in normal culture medium containing either drug vehicles or a low and a high but nontoxic level of glyburide (0.5 and 2.5 $\mu$mol/L), pioglitazone (1 and 5 $\mu$mol/L), and metformin (20 and 100 $\mu$mol/L). Vehicle-treated cells increased from 2 to 9 to 14 (cells per well $\times 10^3$; 5 wells each) from day zero to 4 to 9 to 14. From day 9 to 14 these cell numbers were decreased an average of 20% by the 2.5 $\mu$mol/L glyburide ($P < .05$) and 43% by the 5 $\mu$mol/L pioglitazone ($P < .05$). The low levels of glyburide and pioglitazone and both the low and high levels of metformin failed to influence cell numbers. In a second experiment, even half the abovementioned high level of pioglitazone (2.5 $\mu$mol/L) still exerted a markedly greater antiproliferative effect on aortic SMC than a high level of 2.0 $\mu$mol/L of glyburide ($P < .05$). In addition, neither drug's antiproliferative effect was influenced by a high level of insulin added to the medium (10 mU/mL). Similarly, a small but significant stimulatory effect of this high insulin on cell proliferation ($P < .05$) was not significantly affected by these two drugs (although pioglitazone tended to inhibit it). These results suggest that thiazolidinediones may be more useful antidiabetic agents than sulfonylureas and biguanides in inhibiting abnormal arterial SMC proliferation associated with atherosclerosis and postangioplasty restenosis which are common in diabetic patients. Am J Hypertens 1996;9:188–192

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on arterial smooth muscle cell proliferation in culture. Metformin appeared to greatly inhibit proliferation of a population of human arterial smooth muscle cells grown for 7 days in culture medium containing 5% serum collected from either diabetic or nondiabetic patients. Recently, the thiazolidinedione pioglitazone (an experimental antidiabetic agent) was shown to substantially inhibit proliferation of smooth muscle cells derived from renal arteries of normal rats and grown for 14 days in medium containing 5% fetal calf serum. Therefore, the present experiments were conducted to not only confirm these findings but also to compare direct effects of glyburide (a sulfonylurea), metformin (a biguanide), and pioglitazone (a thiazolidinedione) on proliferation of a population of arterial smooth muscle cells, all obtained from the same source and all grown in the same culture medium at the same time.

Our cells were derived from explanted aortic tissues of Dahl salt-sensitive (S) rats. Smooth muscle cells cultured from explants of large arteries have been especially useful in the investigation of atherosclerotic mechanisms as they are more similar phenotypically to abnormally proliferating smooth muscle cells in atherosclerotic lesions than cells derived by enzymatic dissociation. The Dahl S rat is an insulin-resistant and dyslipidemic model of hypertension in which pioglitazone significantly enhances proliferative growth of these cells. Other drugs that inhibit proliferation of arterial smooth muscle cells in culture (eg, calcium channel antagonists) also reduce atherosclerotic lesions in vivo in Dahl S rats and do so at less than antihypertensive doses.

Methods
Thoracic aortae from Dahl S rats were stripped of adventitia and cut into multiple ring segments. These rings were cultured as explants in a series of two plastic wells per ring (each ring remaining 1 week in each well) according to a previously described methodology. Mixtures of endothelial and smooth muscle cell outgrowths from rings in the first set of wells were discarded. Smooth muscle cell outgrowth from rings in the second set of wells (with no endothelial cells) were grown to confluence and subcultured through 4 passages to obtain enough for later study. These smooth muscle cells were stored under liquid nitrogen and used as needed for experimentation.

Cells were characterized in their sixth passage as smooth muscle and not endothelial cells by their morphology and immunofluorescent staining. They were also examined for acute cytotoxic effects of glyburide, pioglitazone, and metformin at multiple concentrations of each drug. Then they were seeded at 4 x 10^4 cells per 35 mm well (seventh passage) and after 1 day treated every 2 to 4 days with 5% fetal bovine serum (FBS; Gibco, Grand Island, NY) in normal culture medium (Dulbecco's Modified Eagle's Medium [DMEM]; Gibco) containing experimental agents (nontoxic levels of antidiabetic drugs = human insulin). Attached cells were harvested with trypsin and counted ( Coulter; counter) at selected time intervals over a period of 2 weeks (5 to 6 wells/interval/treatment) at the end of which cell viability (via trypan blue exclusion) was examined again.

In the first of two growth experiments, nontoxic concentrations of all three antidiabetic drugs (0.5 and 2.5 \( \mu \text{mol} / \text{L} \) glyburide, 1 and 5 \( \mu \text{mol} / \text{L} \) pioglitazone, 20 and 100 \( \mu \text{mol} / \text{L} \) metformin) were examined in parallel for effects on cell proliferation. The high level of each drug closely approximates levels reported previously to be not only maximally effective in terms of various activities in vitro in vascular and nonvascular preparations but also nontoxic in vitro and comparable to plasma levels found after oral administration of hypoglycemic doses. In a second experiment, only glyburide (2.0 \( \mu \text{mol} / \text{L} \)) and pioglitazone (2.5 \( \mu \text{mol} / \text{L} \)) were tested, each in the presence as well as in the absence of a high level of human insulin (10 mIU/mL) added to the growth medium in separate wells. The baseline level of bovine insulin in this medium (calculated from the FBS hormone profile provided by Gibco) was negligible by comparison: 0.0003 mIU/mL in this experiment and 0.0001 mIU/mL in experiment 1.

In both experiments, cell numbers were expressed as mean \( \pm \) SE and, after transformation to achieve homogeneity of variance (if necessary), they were evaluated by analysis of variance (ANOVA) followed by several multiple mean comparison procedures to identify significant individual mean differences at \( P < .05 \).
well (×10^4), cells treated with the high level of glyburide numbered 162 ± 5 per well (×10^4) and cells treated with the high level of metformin numbered slightly more than control (239 ± 7 per well × 10^4). In addition, dye exclusion tests with trypan blue indicated no differential effects of these antidiabetic agents on cell viability on day 14. This agreed with our initial acute tests for cytotoxicity which were positive only at levels 5 to 10 times higher than the high levels employed in this long term experiment.

In the second growth experiment (Figure 1) we found that even half the high level of pioglitazone used in experiment 1 (2.5 μmol/L) arrested nearly all rapid proliferative growth of these Dahl S aortic smooth muscle cells in response to growth medium containing a new batch of FBS. A level of glyburide similar to the high level used in experiment 1 (2.0 μmol/L) only partially attenuated such growth. In this second experiment, a high level of insulin added to the medium in separate wells slightly but significantly stimulated cell proliferation. Two-factor ANOVA revealed no statistically meaningful interaction between the insulin and the drugs in this experiment. In other words, the large and differential inhibitory effects of these drugs on cell proliferation were not significantly influenced by the high insulin and the small stimulatory effect of this high insulin was not significantly influenced by the drugs (although pioglitazone tended to inhibit it). As in experiment 1, there were no effects of drugs (or insulin) on cell viability at the conclusion of this experiment.

**DISCUSSION**

There are at least two important findings from the present study. First, antidiabetic drugs may differ markedly in their ability to inhibit proliferation of arterial smooth muscle cells in culture. Our results indicate that the thiazolidinedione pioglitazone inhibits the rapid proliferation characteristic of smooth muscle cells derived from rat aortic explants to a much greater extent than the sulfonylurea glyburide and certainly the biguanide metformin. Second, this inhibitory action of pioglitazone and even the lesser inhibitory action of glyburide do not appear to significantly affect the ability of insulin to stimulate proliferation of these cells. Nor does insulin affect the inhibitory actions of the drugs.

Our work confirms a previous demonstration by Dubey et al. of the antiproliferative potential of pioglitazone in rat arterial smooth muscle cells grown in medium containing 5% fetal calf serum. However, we were unable to confirm the antiproliferative effect of metformin previously reported by Bünting et al. The reasons for this may be threefold. First, the arterial smooth muscle cells treated with high levels of pioglitazone and metformin showed no effects of drugs on cell viability at the conclusion of this experiment.

**GROWTH OF AORTIC SMOOTH MUSCLE CELLS ISOLATED FROM DAHL SALT-SENSITIVE RATS**

**CELLS INCUBATED IN 5% FBS**

- Glyburide (1 μg/ml)
- Pioglitazone (1 μg/ml)
- Control (vehicle)

**CELLS INCUBATED IN 5% FBS + INSULIN (10 mU/ml)**

**DAYS OF TREATMENT**

**FIGURE 1.** Effects of glyburide and pioglitazone on proliferative growth of Dahl S rat aortic smooth muscle cells in normal culture medium containing 5% fetal bovine serum (FBS) with and without added insulin. Cells were seeded into passage 7 at 4 × 10^4 cells/35 mm well and fed experimental media 24 h later and at 2 to 3 day intervals thereafter. Statistically significant inhibitory effects of the drugs (pioglitazone v glyburide v control, P < .05) and a stimulatory effect of insulin (P < .05) was detected from day 8 to day 14 (evaluated by ANOVA and multiple mean comparisons). Over the same period, there was no statistically significant interaction between the effect of the drugs and the effect of insulin (two-factor ANOVA). Thus, although the stimulatory effect of insulin appeared to be less in the presence of pioglitazone there was no statistical support for this phenomenon in the present study.
smooth muscle cells employed by Bunting et al were described as human in origin (although no particular vessel or method of primary culture was specified). Our cells were rat in origin obtained specifically from aorta by the explant method. Second, the growth serum employed by Bunting et al was derived from diabetic and nondiabetic patients. They reported that the antiproliferative action of metformin was independent of the serum. Nonetheless, because the serum we employed (fetal bovine serum) is not human serum, our effects of metformin may be different for that reason. Finally, we did not test metformin at levels above 100 μmol/L. Bunting et al only reported notable antiproliferative activity of metformin at much higher levels (1,000 to 40,000 μmol/L). However, they also reported cell death at high levels of metformin. Therefore, had we raised metformin above 100 μmol/L we might have seen a significant antiproliferative effect as well but possibly related to an undesirable, nonspecific toxic action. Whether metformin can exert a potentially beneficial antiproliferative effect by way of a nontoxic mechanism remains to be more fully explored.

Mechanisms responsible for the inhibitory effects of glyburide and pioglitazone on proliferation of the Dahl S aortic smooth muscle cells in the present study also remain to be determined. This study suggests that these two different agents do not inhibit proliferation of this particular cell line by interacting with the ability of insulin to stimulate proliferation. They may inhibit proliferation as induced by other trophic factors present in the growth serum. Fetal bovine serum normally contains substantial quantities of platelet-derived growth factor (PDGF). Ciglitazone inhibits sustained increases in intracellular free calcium induced by PDGF in cells derived from vascular smooth muscle and other tissues. Thus, pioglitazone and its thiazolidinedione analogs may inhibit aortic smooth muscle cell proliferation by interfering with trophic mechanisms of PDGF. This may also explain why a somewhat lower concentration of pioglitazone in our second experiment inhibited proliferation more than at a higher concentration in our first experiment. We used a new batch of fetal bovine serum in our second experiment which may well have been different in terms of the concentration of PDGF.

There are a number of important therapeutic implications associated with these antiproliferative actions of glyburide and pioglitazone. Arterial smooth muscle cell proliferation is known to contribute importantly to atherosclerosis and may also contribute to hypertension. Both diseases are common in insulin-resistant diabetic subjects and are also found to be associated with insulin-resistance in nondiabetics. Thus, there is considerable interest in whether actions of pharmacological agents with insulin-sensitizing properties include antithrombotic and antihypertensive as well as antihyperglycemic potential. The present study suggests that pioglitazone could at least inhibit abnormal proliferation of arterial smooth muscle cells in atherosclerotic lesions to a greater extent than glyburide and metformin. Pioglitazone also decreases intestinal absorption and therefore plasma concentrations of cholesterol and other lipids in animals fed high cholesterol. Thus, pioglitazone and other thiazolidinediones also decrease blood pressure chronically in hypertensive animals and they do not stimulate insulin secretion. Whether they actually decrease incidence or severity of atherosclerotic lesions in vivo and to a greater extent than sulfonylureas and biguanides remains to be determined. Such in vivo studies should include effects on restenotic lesions induced by arterial balloon angioplasty. Restenosis after angioplasty occurs more frequently in diabetics than nondiabetics and arterial smooth muscle cell proliferation may play an even more important role in postangioplasty restenosis than in primary atherosclerosis.

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