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EVIDENCE THAT PIOGLITAZONE INCREASES INTRACELLULAR FREE MAGNESIUM CONCENTRATION IN FRESHLY ISOLATED RAT ADIPOCYTES

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Pioglitazone is one of a new class of oral agents being developed as antidiabetic agents which can increase insulin sensitivity, reduce blood pressure and improve abnormalities in lipid metabolism. The mechanism of action of pioglitazone is not known. Increasing evidence suggests that magnesium can play an important role in regulating glucose homeostasis and vascular tone. To clarify a potential mechanism of pioglitazone action we determined the effects of pioglitazone on intracellular free magnesium concentration in freshly isolated rat adipocytes. Concentrations of pioglitazone as low as 300 nM markedly increased the free magnesium concentration in the adipocytes. Pioglitazone action was selective for magnesium since intracellular free calcium concentration was not altered. These new data suggest that a potentially important mechanism explaining the metabolic and vascular actions of these new anti-diabetic agents is increasing free magnesium concentration in target tissues.

Pioglitazone, a thiazolidine derivative, (5-[4-(2-(5-ethyl-2-pyridyl)ethoxy)benzyl]-2,4-thiazolidine dione) is one of a new class of compounds showing promise as an antidiabetic agent (1,2). Pioglitazone and other members of this class of compounds lowers plasma glucose

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levels, as well as insulin and triglyceride levels in models of human non-insulin dependent diabetes (NIDDM) in which insulin resistance is a major metabolic defect (3,4). Recent data also reveals similar action of these agents in human NIDDM (5). The major effect of these agents is to improve the post-receptor binding action of insulin in target tissues (1,6-8). It is of interest to note that the thiazolidine compounds can also reduce blood pressure in certain animal models and reduce intracellular calcium concentration (9). However, the precise mechanism of action of pioglitazone and other thiazolidine derivatives remains unclear.

Increasing evidence suggests that magnesium (Mg) plays an important role in the regulation of vascular tone and glucose homeostasis (10-13). Mg deficiency has been noted to produce insulin resistance in humans (10). Furthermore, intracellular free Mg deficiency has been recently noted to be a prominent feature of NIDDM (14). Recent data reveals Mg supplementation can improve insulin responsiveness in patients with NIDDM (15). In addition, Mg administration can reduce blood pressure, triglyceride levels and inhibit the actions of angiotensin II (16-19). The purpose of the present study was to evaluate whether pioglitazone can alter intracellular free Mg concentrations in rat adipocytes in order to elucidate a potential mechanism explaining the actions of pioglitazone on both vascular tone and glucose homeostasis.

Materials and Methods

Isolation of adipocytes

Fat cells were prepared essentially by the collagenase digestion method of Rodbell (20). Epididymal fat pads from fed Sprague/Dawley rats (Charles River, Wilmington, MA) were minced, incubated in a Krebs-Ringer bicarbonate buffer containing 4% BSA, 5.5 mM glucose, 2.5 mM calcium chloride and collagenase at 2 mg/ml.

The incubation was for 60 min at 37°C, under a 95% 0_2, 5% CO_2 atmosphere with orbital shaking. After 60 minutes any remaining clumps were broken up by gentle aspiration and the cells were washed two times in Krebs-Ringer/BSA minus collagenase and one time in incubation medium which was Gibco/BRL DMEM (Gaithersburg, MD) + 1% BSA, 0.4% FBS, 5.5 mM glucose, 1% penicillin/streptomycin, 10 mM HEPES pH 7.4, normalized for magnesium and calcium. The fat cells were then counted and suspended in fresh incubation media at $1x10^6$/mL and incubated for 1 hour at 37°C in 5% CO_2, 95% air to equilibrate.

After equilibration, aliquots of fat cells ($5x10^5$/mL) were treated with pioglitazone in DMSO, DMSO alone, buffer & insulin or buffer alone for the indicated times. All studies were approved by the City of Hope Animal Committee. Pioglitazone was kindly provided by Dr. Jerry Colca at the UpJohn Co., Kalamazoo, MI.

Fluorescent dye loading and $[Ca^{2+}]_i$ and $[Mg^{2+}]_i$ assay of fat cells

After the drug or control incubations, the fat cells were washed in HEPES-Tyrode buffer (HEPES 10 mM pH 7.6; NaCL, 145 mM; KCl, 5 mM; NaH_2PO_4, 0.5 mM; dextrose, 5.5 mM; BSA 0.1% filter sterilized and corrected to pH 7.4).

The cells were then loaded at $5x10^5$ cells/mL in HEPES/tyrode buffer + mag fura-2AM or Fura-2AM (Molecular Probes, Eugene, Oregon) at 2-4 uM, incubated at 37°C for 30 minutes in the dark. After this time, the cells were diluted to $1X10^5$/ml and incubated a further 30 minutes at 37°C in the dark. The cells were then washed two times in HEPES-tyrode buffer

and suspended at $5x10^5$ cells per ml in HEPES=tyrode buffer. The cells were then assayed for $[Ca^{2+}]_i$ or $[Mg^{2+}]_i$ in a Hitachi f-2000 fluorescence spectrophotometer as described previously (21) at ex 335-370 em 510 for $[Mg^{2+}]_i$ and ex 340-380 em 510 for $[Ca^{2+}]_i$. Ionic concentrations were calculated as described (21). Results are reported as mean ± SE. Significance was determined using the student's t test.

Results

We determined the dose and time dependent effects of pioglitazone on $[Mg^{2+}]i$ in freshly prepared isolated rat adipocytes. As shown in table 1, pioglitazone at concentrations as low as 300 nM significantly increased the concentration of free Mg in these adipocytes. These effects were also evident at higher concentrations (2.5 mM). The effects of pioglitazone to increase $[Mg^{2+}]i$ could be demonstrated as early as 2 minutes (data not shown). Furthermore, pioglitazone particularly at higher concentrations also increased free Mg concentration in adipocytes incubated for up to 4 hours (180±23 percent of control, $p<0.05$, n=3).

Data from a representative experiment showing the changes in $[Mg^{2+}]i$ and $[Ca^{2+}]i$ using pioglitazone at 5 minutes is shown in figure 1. The results demonstrate over a 2 fold increase of $[Mg^{2+}]i$ at 625 nM concentration of pioglitazone without any change in $[Ca^{2+}]i$. The effect of pioglitazone on $[Mg^{2+}]i$ was also evaluated in cells incubated for 24 hours. In these studies pioglitazone had no definitive effect on [Mg]i (data not shown).

In other experiments we further evaluated the effect of pioglitazone at 625 nM concentration on free Ca^{2+} concentration in freshly isolated rat adipocytes. Unlike it's effect on $[Mg^{2+}]i$, pioglitazone had no significant effect on Ca^{2+} in these cells (127 ± 13.9 nM basal vs 119 ± 15 nM and 106 ± 3 nM for pioglitazone 625 nM at 5 minutes and 60 minutes incubation time resp.).

The results of this study demonstrate a potent effect of the new antidiabetic agent pioglitazone to selectively increase free Mg^{2+} concentration in rat adipocytes. This is the first

Table 1. Time and Dose Effects of Pioglitazone on $[Mg^{2+}]i$ in freshly isolated rat adipocytes

	Percent of Control		
Dose Pioglitazone	Time (minutes)		
(nM)	5	10	60
0	100	100	100
300	144±20*	112±7	100
625	174±11*	123±7*	158±26
2500	194±66*	124±20	184±41*

Data shown as percent of control represent mean ± SE from 3-5 experiments, *$p < 0.05$.

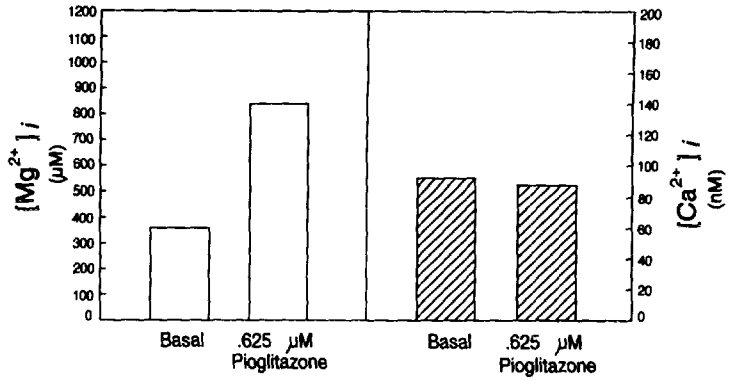

Fig. 1. Effect of pioglitazone on intracellular free Mg^{2+} and Ca^{2+} concentration in fresh adipocytes. $[Mg^{2+}]i$ and $[Ca^{2+}]i$ were separately determined using Mag Fura 2 or Fura 2 as described in methods. A representative experiment is shown comparing the effects of pioglitazone at .625 μM concentration on $[Mg^{2+}]i$ and $[Ca^{2+}]i$ at 5 minutes incubation time. Data were derived from a pooled sample of freshly isolated rat adipocytes from 4 rats.

demonstration showing that a synthetic drug can increase cellular free Mg concentration. The concentrations of pioglitazone used in the current report are consistent with concentrations used in other studies showing effects of pioglitazone to increase insulin sensitivity and insulin kinase activity (1,2). The mechanism of how pioglitazone increases $[Mg^{2+}]i$ in rat adipocytes cannot be determined from the current report. However, preliminary results using a Mg free extracellular buffer indicate that at least part of pioglitazone action is to increase Mg^{2+} transport from the extracellular space into the adipocyte. This mechanism would be similar to the action of insulin which has been demonstrated to produce a selective increase in Mg^{2+} transport via the insulin receptor (21).

The precise mechanism of action of pioglitazone to improve glucose homeostasis has not been previously demonstrated. However, it is clear that this novel agent primarily improves insulin action by a post-binding receptor mechanism. The agents are not direct insulin receptor mimetics in isolated membrane preparations but appear to require a cellular component to increase insulin receptor kinase, glucose transporter levels and glycogen synthase activity.

Reduced Mg concentration has been implicated as a factor leading to a post-receptor binding reduction in insulin sensitivity (11). Reductions in extracellular Mg in isolated skeletal muscle reduces insulin-mediated glucose transport (22,23). Furthermore, recent data has shown that isolated dietary induced Mg deficiency in humans leads to a reduction in insulin mediated glucose disposal as reflected by the Bergman minimal model technique (10). Mg supplementation clearly can improve insulin sensitivity in humans with NIDDM (13,15) and

very recent data shows that Mg supplementation can completely prevent dietary fructose-induced insulin resistance in rats (24). Therefore, it is possible that one mechanism of pioglitazone action is to increase the free Mg concentration in insulin sensitive tissues. Consistent with this hypothesis is data showing that pioglitazone completely restores to normal the reduced serum ionized Mg concentration and insulin resistance associated with high fructose-diet feeding (unpublished observation). Additional studies to directly test the role of Mg in pioglitazone metabolic actions will be needed to fully evaluate this hypothesis.

Recent observations demonstrate that thiazolidine compounds can also lower blood pressure in obese Zucker rats and in Dahl salt-sensitive rats both of which have been reported to be insulin resistant (9,25). However, pioglitazone can also reduce blood pressure in rats which are not insulin resistant such as rats with renovascular hypertension (25). In this model pioglitazone reduces peripheral vascular resistance and increases cardiac output suggesting it is a vasodilator (25). The present report did not directly address the role of pioglitazone induced Mg^{2+} transport on the vascular effects of this compound. However, it is well established that Mg^{2+} produces vasodilation in vitro and in humans (12,16,18,19). Furthermore, Mg^{2+} action in part involves reducing the concentration of free Ca^{2+} in vascular and other tissues (16,26). Therefore, it is certainly plausible that pioglitazone induced increases in free Mg^{2+} concentration may be an important mechanism for pioglitazone mediated reductions in vascular tone. However, additional studies will be required in vascular tissues to explore this hypothesis.

In summary, we have shown for the first time that pioglitazone is a potent stimulator of free Mg^{2+} concentration in adipocytes. These results provide a potentially important mechanism explaining the beneficially actions of pioglitazone on glucose homeostasis and vascular tone.

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