Evidence of direct smooth muscle relaxant effects of the fibrate gemfibrozil

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Abstract

Fibrates are commonly employed to treat abnormal lipid metabolism via their unique ability to stimulate peroxisome proliferator-activated receptor alpha (PPARα). Interestingly, they also decrease systemic arterial pressure, despite recent evidence that PPARα may contribute to expression of renin and related hypertension. Yet, mechanisms responsible for their potential antihypertensive activity remain unresolved. Rapid decreases in arterial pressure following bolus intravenous injections of bezafibrate strongly suggest they may relax arterial smooth muscle directly. But since bezafibrate is highly susceptible to photodegradation in aqueous media, it has never been critically tested for this possibility *in vitro* with isolated arterial smooth muscle preparations. Accordingly, we tested gemfibrozil which is resistant to photodegradation. We examined it over a therapeutically-relevant range (50–400 μM) for both acute and delayed relaxant effects on contractions of the isolated rat tail artery; contractions induced by either depolarizing its smooth muscle cell membranes with high potassium or stimulating its membrane-bound receptors with norepinephrine and arginine-vasopressin. We also examined these same gemfibrozil levels for effects on spontaneously-occurring phasic rhythmic contractile activity, typically not seen in arteries under *in vitro* conditions but commonly exhibited by smooth muscle of uterus, duodenum and bladder. We found that gemfibrozil significantly relaxed all induced forms of contraction in the rat tail artery, acutely at the higher test levels and after a delay of a few hours at the lower test levels. The highest test level of gemfibrozil (400 μM) also completely abolished spontaneously-occurring contractile activity of the isolated uterus and duodenum and markedly suppressed it in the bladder. This is the first evidence that a fibrate drug can directly relax smooth muscle contractions, either induced by various contractile agents or spontaneously-occurring. These findings are particularly relevant to both the recently renewed concern over the impact of fibrates on hypertension and a new understanding of their gastrointestinal side effects.

Key words: fibrates, smooth muscle, rat

Introduction

Bezafibrate, ciprofibrate, clofibrate, fenofibrate and gemfibrozil are all members of a class of

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therapeutic agents known as fibric acid derivatives (or fibrates) (Miller and Spence, 1998; Remick *et al.*, 2008). With the exception of clofibrate (the prototype, no longer available for clinical use), they are all currently employed for treatment of abnormalities in lipid metabolism, particularly hypertriglyceridemias (Miller and Spence, 1998; Remick *et al.*, 2008). The principal mechanism whereby they modify lipid metabolic pathways involves their ability to stimulate a specific nuclear receptor designated as peroxisome proliferator-activated receptor alpha (PPARα) (Mahley and Bersot, 2006; Remick *et al.*, 2008). Knocking out the gene for PPARα was recently reported to abolish hypertension associated with an overactive human renin-angiotensin-aldosterone system transgenically expressed in mice (Tordjman *et al.*, 2007). Thus, an important question is now being raised, *i.e.* whether fibrates by stimulating PPARα will aggravate related forms of hypertension in humans (Yagil and Yagil, 2007; Kuipers *et al.*, 2008). This is also important because hypertension is so highly prevalent among dyslipidemic patients (particularly those with hypertriglyceridemias) (Criqui *et al.*, 1986; Assmann and Schulte, 1987). Indeed, more than a third of all hypertriglyceridemic patients are also hypertensive; twice the prevalence of hypertension observed among normotriglyceridemic individuals (Assmann and Schulte, 1987). However, thus far only two studies have actually reported elevations in blood pressure associated with fibrate use, one in the abovementioned mouse model of an overactive renin-angiotensin-aldosterone system (Tordjman *et al.*, 2007) and one in a small number of healthy men and women volunteers (Subramanian *et al.*, 2006). Otherwise, three other animal and two other human studies have reported no change in blood pressure (Ogawa and Tasaka, 1995; Kunes *et al.*, 2000; Iglarz *et al.*, 2003; Ansquer *et al.*, 2005; Keech *et al.*, 2005) and a notably larger number (nine animal studies and eight human studies) have reported significant reductions in blood pressure after fibrate treatment (Committee of Principal Investigators, 1978; Cruz *et al.*, 1990; Atarashi *et al.*, 1993; Roman *et al.*, 1993; Goode *et al.*, 1995; Matsui *et al.*, 1997; Agrawal *et al.*, 1998; Wilson *et al.*, 1998; Si *et al.*, 1999; Shatara *et al.*, 2000; Jonkers *et al.*, 2001; Kim *et al.*, 2003; Borghi *et al.*, 2004; Diep *et al.*, 2004; Williams *et al.*, 2005; De *et al.*, 2007; Chew *et al.*, 2008).

Thus, it seems reasonable to conclude that fibrate drugs can activate blood pressure-lowering mechanisms, which in turn are at least capable of offsetting if not completely overriding any potential blood pressure-elevating effects they might have due to their stimulation of PPARα-dependent renin-angiotensin-aldosterone activity. Yet, surprising little attention has been given to the study of their blood pressure-lowering mechanisms. For example, only one study to our knowledge has been conducted to determine if they exert any direct vasodilatory properties in isolated arterial smooth muscle preparations. Several years ago clofibrate was shown to inhibit contractions induced *in vitro* by either norepinephrine, histamine or angiotensin in arterial rings prepared from rabbit aorta (Fairhurst *et al.*, 1981). However, this inhibition only occurred when the concentration of calcium in the incubation medium (Krebs buffer) was markedly reduced to low, subnormal levels. No inhibition was observed at levels of calcium normally present in the circulation (Fairhurst *et al.*, 1981).

But only the prodrug form of clofibrate was tested in this *in vitro* work (Fairhurst *et al.*, 1981), not the active form (clofibric acid) to which the prodrug is immediately hydrolyzed once it enters the body (Mahley and Bersot, 2006). Thus, it still remains to be determined whether an active fibrate drug can directly relax arterial or, for that matter, any other smooth muscle-containing

tissue. Only bezafibrate, ciprofibrate and gemfibrozil are active (as lipid-modifying agents) in the forms in which they are administered for clinical use (Miller and Spence, 1998) and are readily available in those forms for experimental purposes. Oral preparations of both bezafibrate and gemfibrozil have been shown to lower blood pressure chronically in animals and humans (Cruz *et al.*, 1990; Atarashi *et al.*, 1993; Matsui *et al.*, 1997; Agrawal *et al.*, 1998; Si *et al.*, 1999; Jonkers *et al.*, 2001; Kim *et al.*, 2003; Borghi *et al.*, 2004). In addition, bezafibrate has even been reported to do so rapidly, *i.e.* after bolus intravenous injections into either normotensive or hypertensive adult male rats (Agrawal *et al.*, 1998). This argues convincingly for a direct vasodilatory mechanism. Unfortunately, bezafibrate is not particularly suitable for any typical *in vitro* investigation of this phenomenon because it is highly susceptible to photodegradation in aqueous media (Canudas *et al.*, 1996). Thus, we chose to employ gemfibrozil in the present work.

We sought to examine gemfibrozil at multiple therapeutically-relevant concentrations for both immediate and delayed relaxant effects on isolated arterial vascular tissue, *i.e.* intact rings prepared from portions of the ventral tail artery of the rat and contracted with either high potassium (K) buffer, norepinephrine (NE) or arginine-vasopressin (AVP). We also examined gemfibrozil for its ability to relax other smooth muscle-containing tissues, *i.e.* uterus, duodenum and bladder. These tissues are known for their ability to exhibit spontaneously-occurring phasic rhythmic contractions, which are physiologically relevant and can be observed *in vitro* without the need to administer contractile agents (Small and Weston, 1971; Gershon, 1981; Granger *et al.*, 1986; Leroy *et al.*, 1991; Haynes and Pennefather, 1993; Buckner *et al.*, 2002; Vedernikov *et al.*, 2003; Azadzoi *et al.*, 2004; Szigeti *et al.*, 2005; Bulbul *et al.*, 2007; Kanai *et al.*, 2007). We determined if gemfibrozil inhibits these spontaneous activities.

Materials and Methods

Experimental tissues

The following smooth-containing tissues were isolated as needed from adult female Sprague-Dawley rats immediately after euthanasia: uterus, duodenum, bladder and the ventral tail artery. This procedure was approved in advance by the Institutional Animal Care and Use Committee of Midwestern University. As indicated in the Introduction, the uterus, duodenum and bladder were chosen because of their ability to exhibit spontaneously-occurring phasic rhythmic contractions *in vitro*. Like most arteries, the rat ventral tail artery typically does not exhibit such activity *in vitro*. It was chosen because of its wide-spread use as a convenient model for other vessels. Its most distal portion is very similar functionally and structurally to the many resistance vessels throughout the body (small arteries and arterioles) (Sittiracha *et al.*, 1987; Souza *et al.*, 2008). Its most proximal portion is more similar to larger, conductance arteries (Souza *et al.*, 2008).

After removal of surrounding excess adipose and connective tissue, each uterine horn and duodenum was sectioned into longitudinal segments (each 2 centimeters in length) and the bladder was cut in half from the top of the body (dome) to the bottom of the base. Each of these initial segments was then fitted with thin silk sutures to allow for its suspension from an isometric force (tension)-measuring transducer down into a conventional *in vitro* muscle bath. Each portion of the tail artery (distal and proximal) was sectioned into multiple 3-millimeter cylindrical rings, using a

bound set of evenly-spaced scalpel blades to optimize uniformity. Each of these rings was mounted between two tungsten wire stirrups, which are strong enough not to bend during ring contraction yet thin enough not to damage the inner endothelial cell layer. These stirrups allowed for suspension of each ring from a force-measuring transducer down into a muscle bath.

Each *in vitro* muscle bath consisted of 40 mL of standard physiological Krebs buffer, warmed to 37°C and gassed to a pH of approximately 7.4 with a regulated delivery of a 95%/5% mixture of O_2/CO_2. Before administering any experimental agents, all tissues were allowed to equilibrate in such baths for several minutes at the following experimentally-applied basal (resting) tensions (in milligrams = mg units): 500 for each uterine segment, 2,000 for each duodenal and each half-bladder segment, and 1,250 for each tail arterial ring. All tensions for these tissues were recorded (in mg units) with the aid of the abovementioned force transducers connected to an 8-channel Grass paper chart recorder.

Experimental buffers and agents

Our standard physiological Krebs buffer consisted of the following reagents in distilled water (each in mM units): 122 NaCl, 21 $NaHCO_3$, 4.8 KCl, 1.2 KH_2PO_4, 1.6 $CaCl_2$, 1.2 $MgSO_4$, and 10 glucose. A high potassium (K) buffer was also prepared, to contract arterial smooth muscle without activating contractile receptors. The total concentration of its K was 90 mM, achieved by adding more potassium chloride (KCl) to standard Krebs buffer (while lowering NaCl proportionately to maintain isotonicity and keep the chloride concentration the same). Phentolamine (1 μM) was added to this buffer to block any contribution of K-evoked release of endogenous NE (from adrenergic nerve endings) to K-induced smooth muscle contractions. The contractile receptor agonists NE and AVP were prepared in distilled water and gemfibrozil was prepared in dimethyl sulfoxide (DMSO), all in highly concentrated solutions, before administering them (in ≤40 μL volumes) to the 40-mL bath buffers containing the tissues.

Experimental procedures

The following graded concentrations of gemfibrozil were employed to test for its effects on smooth muscle contractions in uterine, duodenal, bladder and tail arterial tissues: 50, 100, 200 and 400 μM. All these levels are therapeutically-relevant. Plasma levels of the drug range in humans from as low as 20 to as high as 240 μM after standard oral dosing (Wen *et al.*, 2001), and concentrations in certain tissues (particularly intestine) are known to exceed plasma levels (Mahley and Bersot, 2006). While all the above levels of gemfibrozil were used to test for acute effects of the drug (in all tissues), only 50 and 100 μM (which exerted little if any acute effects) were used to test for delayed effects (and only in arterial tissues). In each set of experiments, the drug's vehicle (DMSO) was administered by itself to separate tissues, to serve as a zero control condition (0 μM gemfibrozil).

Acute effects of gemfibrozil on spontaneously-occurring contractions in uterus, duodenum and bladder were only followed for 30 min after its administration. For acute effects of gemfibrozil on tail arterial rings, it was necessary to first contract them with contractile agents. Accordingly, we precontracted them by either depolarizing their smooth muscle cell membranes with high K buffer, or activating their smooth muscle membrane-bound receptors with either NE or AVP. For delayed

effects of gemfibrozil on tail arterial rings, they were first pretreated for 3 hours with either 0, 50 or 100 μM gemfibrozil and then contracted with either high K, NE or AVP.

To contract tail arterial rings with high K buffer, we used a concentration of 90 mM K (prepared as described above) which is maximally effective. To contract with NE and AVP, we used two concentrations of each (one submaximal, near each EC_{50} value, and one maximal) to study acute effects of gemfibrozil, and a wide range of multiple levels (10^{-9} to 10^{-4} M NE; 3×10^{-11} to 3×10^{-8} M AVP) to study delayed effects of gemfibrozil. These multiple levels were administered cumulatively (at 2 min intervals immediately following the 3-hour gemfibrozil pretreatments) to produce an entire concentration-response curve for each contractile agent.

Finally, a limited number of additional experiments were conducted to determine if removal of the endothelium altered gemfibrozil's relaxant effects as observed in the rat tail artery. We tested for acute effects of only the 200 μM level of gemfibrozil and delayed effects of only the 50 (versus 0) micromolar level of the drug on endothelium-denuded arterial rings; rings denuded with saponin as described previously (Graser *et al.*, 1988) and contracted in the same manner as the endothelium-intact rings described above.

Analysis of data

Results from this study were evaluated both visually (from chart recordings) and statistically (from numerical data expressed as mean ± SEM). Statistical methods consisted of either one-way or two-way analysis-of-variance (ANOVA) each followed by Bonferroni's multiple mean comparisons (if appropriate) between different gemfibrozil treatment levels. Differences were designated as statistically significant if the probability of random error (P) was less than 5% ($P<0.05$). In all experiments, n values represent the number of tissues evaluated per treatment level.

Results

Effects of gemfibrozil on K, NE and AVP induced contractions of the rat tail artery

None of the rat tail arterial ring preparations of the present study (from either distal or proximal portions of the vessel) exhibited spontaneous phasic rhythmic contractile activity (as seen with uterus, duodenum and bladder). Gemfibrozil did not alter their baseline (resting) arterial tensions but did relax their contractile tensions induced by high K buffer, NE and AVP. Because results were similar for rings prepared from both distal and proximal portions of the tail artery, the data from each are combined in the following presentations.

Of the gemfibrozil concentrations tested for acute effects (50, 100, 200, 400 μM), all but 50 μM immediately relaxed arterial rings precontracted with either high K buffer or submaximally-effective concentrations of NE and AVP (Fig. 1, Table 1). All but 50 and 100 μM immediately relaxed arterial rings precontracted with maximally-effective concentrations of NE and AVP (Table 1). These relaxations were graded and began immediately after drug administration. In a limited number of additional experiments, 200 μM gemfibrozil produced nearly the same immediate relaxant effects in endothelium-denuded arterial rings as seen with administration of 200 μM to the endothelium-intact arterial rings described in Table 1. For example, the acute relaxation of

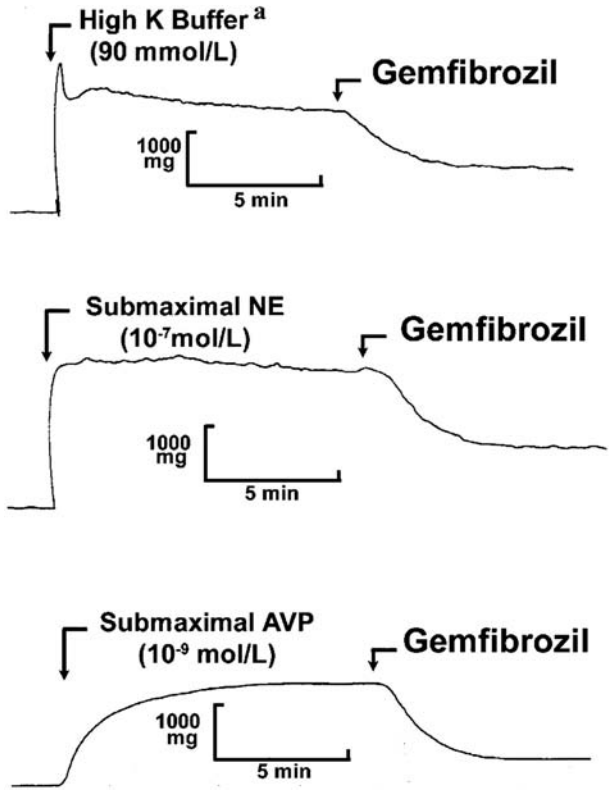

Fig. 1. Representative chart recordings of acute relaxant effects of gemfibrozil (400 μM) on contractions induced by potassium (K), norepinephrine (NE) and arginine vasopressin (AVP) in intact rat tail arterial rings. [a]High K buffer contained 1 μM phentolamine to block contribution of K-evoked release of endogenous NE from adrenergic nerve endings to K-induced contraction.

Table 1. Acute relaxant effects of gemfibrozil on contractions induced by potassium (K), norepinephrine (NE) and arginine vasopressin (AVP) in intact rat tail arterial tissue rings (as illustrated in part in Figure 2)

Contractile Agent	Control Contractile Tension (mg)[b]	% Relaxation of Control Contractile Tension by Gemfibrozil (μM)[c]:				
		0	50	100	200	400
High K Buffer[a] (90 mM/L)	2372 ± 208	4 ± 1	9 ± 2	19 ± 2*	38 ± 2*	59 ± 3*
Submaximal NE (10^{-7} M/L)	2935 ± 161	9 ± 2	11 ± 3	23 ± 3*	35 ± 3*	52 ± 3*
Submaximal AVP (10^{-9} M/L)	2340 ± 114	16 ± 2	23 ± 2	38 ± 3*	53 ± 4*	68 ± 5*
Maximal NE (10^{-4} M/L)	5801 ± 266	–1 ± 2	4 ± 2	7 ± 4	23 ± 4*	42 ± 5*
Maximal AVP (3×10^{-8} M/L)	4304 ± 101	32 ± 2	36 ± 2	40 ± 3	53 ± 3*	70 ± 4*

[a], High K buffer contained 1 micromolar phentolamine to block contribution of K-evoked release of endogenous NE from adrenergic nerve endings to K-induced contraction. [b], Overall mean±SEM (n=40 rings each) observed immediately prior to administration of gemfibrozil (10–11 minutes after administration of contractile agents). For each contractile agent, there were no statistically significant differences between individual control contractile tension means (n=8 rings each) associated with the different gemfibrozil concentrations. [c], Mean±SEM (n=8 rings each) observed 7–8 minutes after administration of gemfibrozil (as illustrated in Figure 2). The vehicle DMSO was administered for the concentration of zero (0) gemfibrozil. *, $P<0.05$ versus mean % relaxation values at all other concentrations of gemfibrozil including zero (as determined by one-way ANOVA followed by Bonferroni's multiple means comparison test).

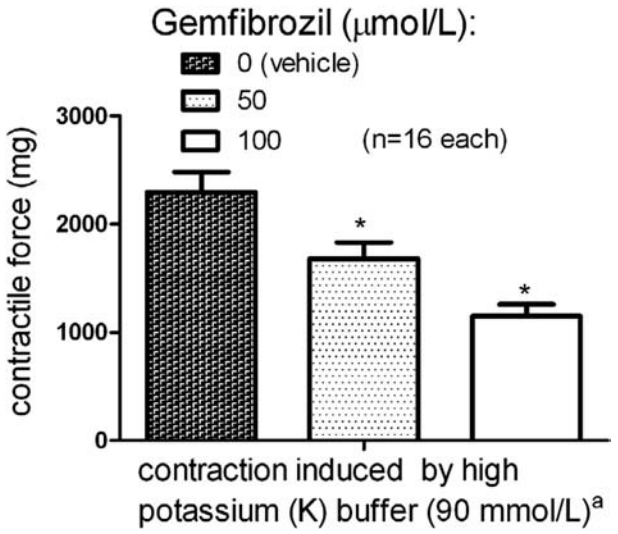

Fig. 2. Delayed relaxant effects of 3-hour pretreatment with 50 and 100 μM gemfibrozil on contractions induced by high potassium (K) in intact rat tail arterial rings (n=16 rings per pretreatment level). Gemfibrozil significantly relaxed the force of K-induced contractions as determined by one-way ANOVA followed by Bonferroni's multiple means comparison test (*, $P<0.05$ versus vehicle). The same statistical analysis also revealed a significantly greater relaxant effect of 100 versus 50 μM gemfibrozil ($P<0.05$). [a]High K buffer contained 1 micromolar phentolamine to block contribution of K-evoked release of endogenous norepinephrine (NE) from adrenergic nerve endings to K-induced contraction.

endothelium-denuded rings precontracted with high K buffer was 39 ± 4% by 200 μM versus no inhibition (3 ± 2%) by zero gemfibrozil ($P<0.05$, n=6 tissues each).

As 50 μM gemfibrozil failed to exert any immediate relaxant effects in the present study and 100 μM failed to do so at least in part (each compared to zero gemfibrozil; Table 1), these two concentrations were re-examined for delayed relaxant effects. Arterial rings pretreated with 50 and 100 μM gemfibrozil for 3 hours showed inhibition of K-, NE- and AVP-induced contractions compared to vehicle-treated rings (Figs. 2–4). These delayed relaxant effects were clearly graded for both K- and NE-induced contractions (*i.e.* relaxant effects of 100 > 50 μM gemfibrozil, Figs. 2 and 3) but not for AVP-induced contractions (Fig. 4). While there was a slight difference between 100 and 50 μM gemfibrozil effects on AVP-induced contractions, it did not achieve statistical significance. Finally, there were no delayed effects of gemfibrozil on basal (resting) arterial tensions. And in a limited number of additional experiments, 3-hour pretreatment with 50 μM gemfibrozil produced nearly the same delayed relaxant effects in endothelium-denuded rings as seen with administration of 50 μM gemfibrozil to the endothelium-intact arterial rings illustrated in Figures 2–4. For example, high K buffer contracted endothelium-denuded rings by only 1,701 ± 162 mg of tension after 3-hour pretreatment with 50 μM gemfibrozil compared to 2,431 ± 226 mg of tension after 3-hour pretreatment with vehicle ($P<0.05$, n=10 tissues each).

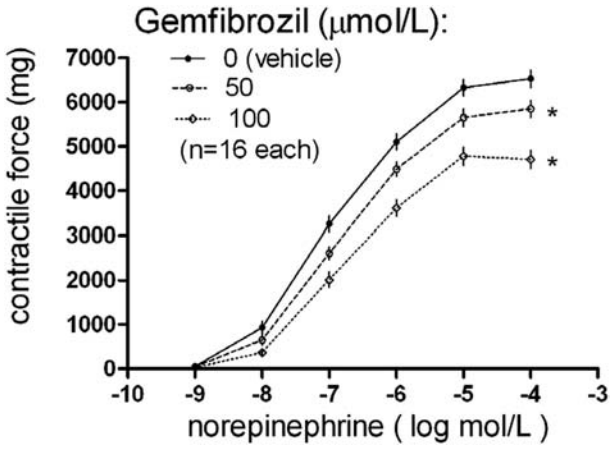

Fig. 3. Delayed relaxant effects of 3-hour pretreatment with 50 and 100 μM gemfibrozil on contractions induced by norepinephrine (NE) in intact rat tail arterial rings (n=16 rings per pretreatment level). Gemfibrozil significantly relaxed the force of NE-induced contraction as determined by two-way ANOVA followed by Bonferroni's multiple means comparison test (*, $P<0.05$ versus vehicle at all levels of NE from 10^{-7} to 10^{-4} M for 50 μM gemfibrozil and from 10^{-8} to 10^{-4} M for 100 μM gemfibrozil). The same statistical analysis also revealed a significantly greater relaxant effect of 100 versus 50 μM gemfibrozil ($P<0.05$) at all levels of NE from 10^{-7} to 10^{-4} M. Gemfibrozil did not significantly alter NE EC_{50} values.

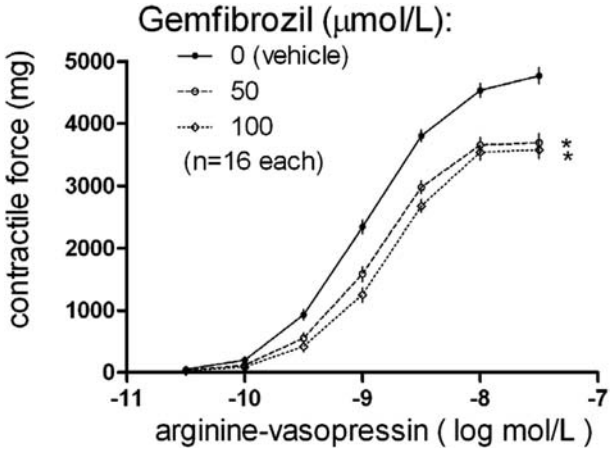

Fig. 4. Delayed relaxant effects of 3-hour pretreatment with 50 and 100 μM gemfibrozil on contractions induced by arginine-vasopressin (AVP) in intact rat tail arterial rings (n=16 rings per pretreatment level). Gemfibrozil significantly relaxed the force of AVP-induced contraction as determined by two-way ANOVA followed by Bonferroni's multiple means comparison test (*, $P<0.05$ versus vehicle at all levels of AVP from 3×10^{-10} to 3×10^{-8} M for both 50 and 100 μM gemfibrozil). The same statistical analysis did not reveal a significantly greater relaxant effect of 100 versus 50 μM gemfibrozil on the force of AVP-induced contraction. Gemfibrozil did not significantly alter AVP EC_{50} values.

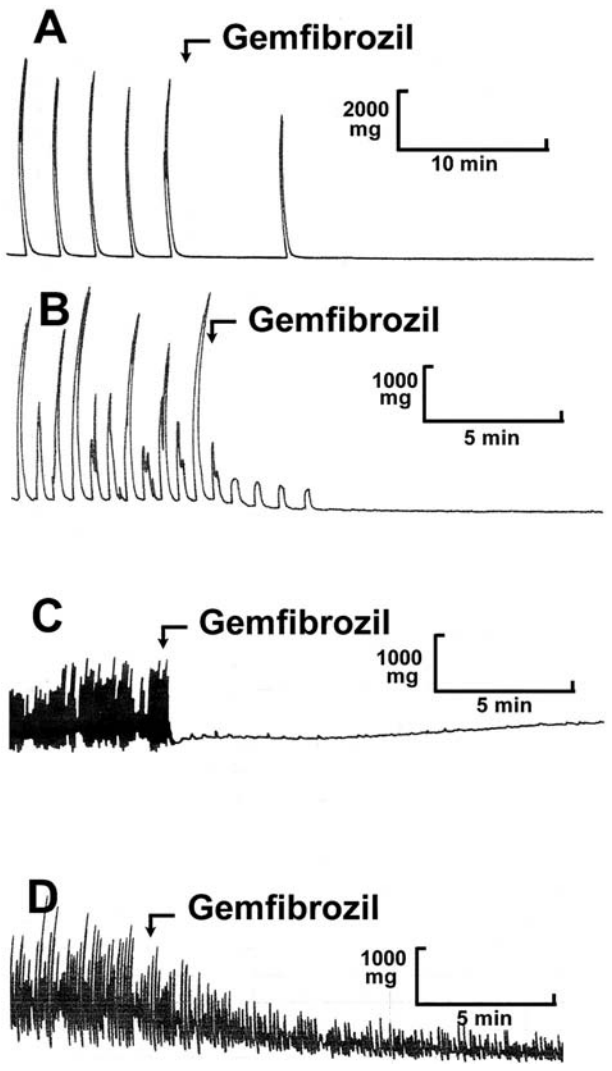

Fig. 5. Representative chart recordings of acute relaxant effects of gemfibrozil (400 μM) on spontaneously-occurring contractions of intact segments of rat uterine horns (A, slowly contracting; B, rapidly contracting), duodenum (C) and bladder (D).

Effects of gemfibrozil on spontaneous contractions of rat uterus, duodenum and bladder

All freshly isolated tissue segments of rat uterus, duodenum and bladder exhibited measurable force (amplitude) and frequency of spontaneous phasic rhythmic contractile activity for at least 1 hour following their suspension in *in vitro* organ baths. Frequencies of such activities varied markedly among individual uterine segments (as typified by the two examples illustrated in Fig. 5, A and B) but not as much among individual duodenal and bladder segments. The following changes in contractile amplitudes were observed after administration of 50, 100, 200 and 400 (versus zero) μM gemfibrozil to these segments. Amplitudes of both the very slowly contracting and the more rapidly contracting uterine segments were inhibited similarly by gemfibrozil; 53 ± 9% by 200 μM

and completely (100 ± 0%) by 400 μM (Fig. 5, A and B) versus no inhibition (2 ± 2%) by zero gemfibrozil ($P<0.05$, n=4 tissues each). Amplitudes of duodenal spontaneous contractions were also inhibited by gemfibrozil; 34 ± 5% and 72 ± 6% by 100 and 200 μM, respectively, and completely (100 ± 0%) by 400 μM (Fig. 5, C) versus no inhibition (–1 ± 2%) by zero gemfibrozil ($P<0.05$, n=4 tissues each). Amplitudes of bladder spontaneous contractions were only inhibited by 400 μM gemfibrozil; 69 ± 8% (Fig. 5, D) versus no inhibition (3 ± 2%) by zero gemfibrozil ($P<0.05$, n=3 tissues each). All these inhibitory actions began immediately and lasted for at least 30 minutes (they were not examined for longer periods). Gemfibrozil did not alter frequencies of spontaneous contractions, except when administered at the 400 μM level to uterine and duodenal segments. Then, because this level completely abolished the amplitudes of spontaneous contractions in those particular tissues (100% inhibition as stated above), it obviously also abolished the frequencies of such contractions in the same tissues (Fig. 5, A–C).

Discussion

Effects of gemfibrozil on K, NE and AVP induced contractions of the rat tail artery

To our knowledge, this study represents the first convincing *in vitro* evidence of a potentially relevant, direct vasorelaxant effect of an active fibrate drug on arterial smooth muscle. As already discussed in the Introduction, the one previous effort to show such evidence (with the inactive prodrug clofibrate) essentially failed to do so, at least in normal physiological media (Fairhurst *et al.*, 1981). It now seems plausible that had clofibric acid (the active drug to which clofibrate is metabolized *in vivo*) been examined instead, that previous *in vitro* effort might have been successful. That would certainly have been more consistent with other previous reports that clofibrate, administered orally to animals and humans, decreases arterial blood pressure *in vivo* (Committee of Principal Investigators, 1978; Roman *et al.*, 1993; Goode *et al.*, 1995; Williams *et al.*, 2005).

The direct vasorelaxant effects of gemfibrozil as seen in the present study are remarkably similar to those we and others have reported previously for the antidiabetic drugs known as thiazolidinediones, *i.e.* troglitazone, pioglitazone and rosiglitazone (Peuler *et al.*, 1997; Ali *et al.*, 1999; Peuler, 2002; Peuler and Phelps, 2004; Peuler *et al.*, 2004). For example, in a previous study, we examined multiple concentrations of the thiazolidinedione troglitazone (2, 4, 8, 16, and 32 μM) for vasorelaxant effects on K-, NE- and AVP-induced contractions in tail arterial tissue rings prepared from the adult male rat (Peuler *et al.*, 2004). As with our effects of gemfibrozil on such contractions in the present study, only the higher concentrations of troglitazone in that prior study were able to relax such contractions immediately; the lower, more therapeutically-relevant concentrations did so only after a delay of a few hours (Peuler *et al.*, 2004). Also, as with gemfibrozil, the relaxant effects of troglitazone did not differ between distal and proximal portions of the rat tail artery and were not influenced by removal of the endothelium (Peuler *et al.*, 2004). The main difference between the two studies was only the source of the tail arterial tissue, male versus female rats.

In one sense, such remarkable similarities are not surprising. Thiazolidinediones were originally synthesized as derivatives of the fibrates (Kalaitzidis *et al.*, 2009). In the process, they

lost much of the lipid-metabolizing properties of the fibrates (as mediated by PPARα) and acquired glucose-metabolizing properties, for which they are now widely-recognized (and which are mediated via another nuclear PPAR, *i.e.* PPARγ) (Kalaitzidis *et al.*, 2009). Like PPARα, PPARγ was also recently reported to play a stimulatory role on expression of the renin-angiotensin-aldosterone system (Todorov *et al.*, 2007; Weatherford *et al.*, 2007). Therefore, like the fibrates (PPARα agonists), the thiazolidinediones (PPARγ agonists) are also now under scrutiny for possible blood pressure-elevating effects via increased renin-angiotensin-aldosterone activity (Todorov *et al.*, 2007; Weatherford *et al.*, 2007; Kuipers *et al.*, 2008). However, all efforts thus far to uncover evidence of thiazolidinedione-induced pressure elevations have failed. Rather, as repeatedly reviewed (Sarafidis and Nilsson, 2006; Giles and Sander, 2007; Kelly and Bank, 2007; Sarafidis, 2008; Takahashi and Kushiro, 2008; Kalaitzidis *et al.*, 2009), many thiazolidinedione studies show decreases in blood pressure for which the abovementioned thiazolidinedione vasorelaxant action is considered a primary mechanism (Kalaitzidis *et al.*, 2009). Thus, like the previous demonstrations of thiazolidinedione-induced vasorelaxation (Peuler *et al.*, 1997; Ali *et al.*, 1999; Peuler, 2002; Peuler and Phelps, 2004; Peuler *et al.*, 2004), our present identification of fibrate-induced vasorelaxation adds an important new dimension to the current concern surrounding stimulatory effects of PPAR agonists in general on renin-angiotensin-aldosterone-related hypertension in humans. Clearly, further in-depth examination of direct vascular effects of gemfibrozil (and other fibrates) is now warranted in this context. The same can be said for a new class of experimental agents currently under development; agents known as dual PPARα/γ agonists (Grether *et al.*, 2009). It is hoped that these agents will combine the beneficial properties of thiazolidinediones and fibrates in one chemical structure (Staels and Fruchart, 2005; Schuster *et al.*, 2008; Henry *et al.*, 2009). Two of these dual agents have already been reported to lower blood pressure in hypertensive animals (Mamnoor *et al.*, 2006; Liao *et al.*, 2009). Mechanisms responsible for this action have not been identified. But now, together with the abovementioned previous thiazolidinedione findings, our present results with the fibrate gemfibrozil clearly provide strong evidence to suspect that a direct vasorelaxant mechanism may play a role in this potential antihypertensive action.

As with gemfibrozil's relaxation of spontaneously-contracting uterus, duodenum and bladder (discussed below), we suspect that the primary site of its relaxation in the arterial wall is most likely the smooth muscle. It is not likely the inner endothelial cell layer, even though long-standing fibrate therapy in both dyslipidemic and diabetic patients has been shown to protect it (Kovacs *et al.*, 2005; Otsuki *et al.*, 2005; Kilicarslan *et al.*, 2008). In the present study, experimental removal of the endothelium did not abolish gemfibrozil's vasorelaxant effects. This does not rule out the possibility of a minor, secondary, modulatory action of the drug via the endothelium. Indeed, the same could be said for the arterial wall's adrenergic nerve endings, which richly innervate the smooth muscle of the rat tail artery (Sittiracha *et al.*, 1987). However, if these nerve endings were the primary site of gemfibrozil's action (*i.e.* either altering release and/or reuptake of NE) then we would not expect it to relax contractions induced by a high K buffer containing the NE receptor blocker phentolamine, or contractions induced by the nonadrenergic peptide AVP which is neither released nor taken up by such nerve endings.

Mechanisms responsible for gemfibrozil's relaxant action on the arterial smooth muscle were

not specifically addressed in the present study. We suspect they may be similar to those responsible for the abovementioned thiazolidinedione-induced vasorelaxations. If so, that would include (in part) a direct inhibition of smooth muscle membrane-bound voltage-operated calcium channels, as previously established for all thiazolidinedione agents through whole-cell patch-clamp techniques (Zhang *et al.*, 1994; Nakamura *et al.*, 1998; Eto *et al.*, 2001). This would be highly consistent with the ability of gemfibrozil in the present study to inhibit contractions induced solely by depolarizing smooth muscle cell membranes with a high concentration of extracellular K. Such high K only contracts smooth muscle by activating those particular channels (Kravtsov and Kwan, 1995). But receptor agonists like NE and AVP are commonly known to activate more smooth muscle contractile mechanisms than that activated by a high concentration of extracellular K (Chen and Rembold, 1995; Kostrzewska *et al.*, 2000; Fallet *et al.*, 2005) and there is convincing evidence that the thiazolidinediones oppose these additional mechanisms. For example, there is evidence that thiazolidinediones open membrane-bound voltage-operated K channels (Peuler *et al.*, 2004) and inhibit receptor-mediated release of calcium from intracellular sarcoplasmic reticulum (SR) storage sites (Ali *et al.*, 1999; Peuler, 2002). The same may be true for gemfibrozil and remains to be determined in future efforts beyond the scope of the present study.

Future efforts may also be necessary to ascertain mechanisms responsible for the delay of a few hours in the vasorelaxant effects of the lower test concentrations of gemfibrozil (*e.g.* 50 μM) in the present work. Most fibrates are fairly lipid soluble (Miller and Spence, 1998; Mahley and Bersot, 2006) and thus should reach any potential intracellular cytosolic sites of action rather quickly. However, if like certain other lipophilic agents, fibrates must enter the space between bilayers of cellular membranes and then move laterally to reach delayed sites of action (Meisheri *et al.*, 1993), a delay in their action seems reasonable. A more likely possibility is that, within a matter of hours, gemfibrozil is able to accumulate in arterial tissue up to concentrations that exceed its extracellular level. All fibrates are already known to do so in liver and kidney after standard oral dosing in humans (Mahley and Bersot, 2006). This could result in delayed actions which simply mimic those that otherwise occur immediately upon exposure to higher extracellular levels. A third possibility is that the delay is related to a fibrate-induced change in PPARα activity within the vascular smooth muscle cell. Because this would involve gene transcription, a delayed change in cell contractile responsiveness would seem reasonable. However, a few hours might not be long enough for such a change. The onset of gemfibrozil's PPARα-dependent lipid-altering actions in dyslipidemic patients requires several days even though its peak plasma level is reached within 1–2 hours after standard oral dosing (Anonymous, 2009). Even under cell culture conditions, PPARα-related changes in vascular smooth muscle cell proliferation rates require at least a few days to begin (Gizard *et al.*, 2005). We suspect this same consideration rules out involvement of PPARγ in our previously reported delays associated with vasorelaxant effects of the thiazolidinediones (Peuler *et al.*, 1997; Peuler *et al.*, 2004).

One phenomenon not previously reported with thiazolidinediones but observed in the present study is an unusual concentration-dependent difference between gemfibrozil's delayed relaxations of AVP- versus NE- and K-induced contractions. The drug's delayed relaxations of both NE- and K-induced contractions were clearly greater at 100 versus 50 μM (Figs. 2 and 3). This was not the case for AVP-induced contractions, particularly at the higher concentrations of AVP (Fig. 4). The

reason for this phenomenon is not readily apparent. But there are known qualitative differences between nonpeptide and peptide related contractions in arterial smooth muscle, including distinct differences between NE and AVP contractions in the rat tail artery (Somlyo *et al.*, 1966; Fox *et al.*, 1992). These differences include 1) a different sensitivity to the impact of magnesium on AVP and adrenergic receptor affinities (Somlyo *et al.*, 1966), 2) a different sensitivity of AVP and NE contractions to metabolic acidosis and uremia (Fox *et al.*, 1992), and 3) a different relationship of AVP and NE contractions to intracellular inositol phosphate levels (Fox *et al.*, 1992). The latter very likely involves the role of inositol phosphates in receptor-induced release of calcium from intracellular SR stores (Blaustein *et al.*, 2002). Conceivably, gemfibrozil may be interacting at the level of any one of these differences, although the latter seems most plausible. Release of calcium from the SR is more important to contractions at higher versus lower concentrations of both NE and AVP (Chen and Rembold, 1995; Kostrzewska *et al.*, 2000; Fallet *et al.*, 2005), and it is at the higher concentrations of these physiologic contractile substances that the difference in concentration-dependence for gemfibrozil's delayed relaxant effects is most obvious (compare Figs. 3 and 4).

Effects of gemfibrozil on spontaneous contractions of rat uterus, duodenum and bladder

To our knowledge, this study provides the first evidence that a fibrate drug can directly inhibit the force of spontaneously-occurring phasic rhythmic smooth muscle contractions in tissues known to normally exhibit such activity (*i.e.* uterus, duodenum and bladder). We did not examine smooth muscle cell layers from these tissues in isolation. Rather, other cell types remained present. In our opinion, it was important that these tissues remain fully intact in an initial preliminary investigation of this sort. Those other cell types include the endometrium which lies inside the myometrial smooth muscle of the uterus, the enteric nervous system which innervates the intestinal smooth muscle of the duodenum, and the urothelium which separates the detrusor smooth muscle of the bladder wall from the urine inside. But while these nonmuscular cells may be able to modulate spontaneous contractility of the adjacent smooth muscle cell layers (Gershon, 1981; Granger *et al.*, 1986; Haynes and Pennefather, 1993; Buckner *et al.*, 2002; Azadzoi *et al.*, 2004; Bulbul *et al.*, 2007; Kanai *et al.*, 2007), they are not likely the primary site for gemfibrozil's relaxant effects. If, for example, gemfibrozil's relaxation of the duodenal contractility seen in Figure 5 were mediated solely by inhibition of enteric neuronal release of contractile neurotransmitters, then we would not expect to see its relaxant influence in uterus and bladder as well. The same argument would apply if gemfibrozil acted solely on either the endometrium of the uterus or the urothelium of the bladder. Thus, we conclude that gemfibrozil's primary site of action is the smooth muscle itself in each tissue, either inhibiting its responsiveness to endogenous contractile substances and/or its inherent ability to contract spontaneously on its own. However, this does not rule out the possibility of minor, secondary, modulator influences of the drug via these other cell types. Future efforts to unravel mechanisms responsible for gemfibrozil's action should be focused on both.

Gemfibrozil's marked inhibition of duodenal spontaneous activity may be particularly relevant clinically. If this inhibitory action occurs *in vivo* and perhaps in the form of a more generalized suppression of gastrointestinal (GI) motility, it could represent the first reasonable explanation for

common GI side effects reported by gemfibrozil-treated patients, *i.e.* abdominal pain, dyspepsia, nausea, vomiting and constipation (Muscari *et al.*, 2002; Remick *et al.*, 2008). The lowest test concentration at which we observed gemfibrozil's inhibition of duodenum was 100 μM, well within the range circulating in plasma of gemfibrozil-treated patients(Wen *et al.*, 2001). However, even if we had only observed this effect at our highest test level (400 μM), it would still be relevant clinically. The GI tract in such patients is typically exposed to concentrations of the drug that notably exceed plasma levels after standard oral dosing (Mahley and Bersot, 2006). Since the same cannot be said for uterus and bladder, the clinical relevance of our results with those tissues remains unclear. However, those tissues could still serve as valuable experimental controls in future efforts to better understand gemfibrozil's action in the intestine.

Summary

To our knowledge, this is the first evidence that an active fibrate drug, widely employed to improve lipid metabolism, can directly relax smooth muscle contractions, either spontaneously-occurring, membrane depolarization-induced, or receptor-mediated. These findings may be clinically relevant to both the recently renewed concern over the impact of these drugs on hypertension and a new understanding of the various GI side effects associated with fibrate therapy.

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