Vasodilation of Rat Retinal Microvessels Induced by Monobutyrin

Dysregulation in Diabetes

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Abstract

1-Butyryl-glycerol (monobutyrin) is a simple lipid product of adipocytes with angiogenic activity. Recent studies have shown that the biosynthesis of this compound is tightly linked to lipolysis, a process associated with changes in blood flow. We now present data indicating that monobutyrin is an effective vasodilator of rodent blood vessels using a fluorescent retinal angiogram assay. The vasodilatory activity of monobutyrin is potent ($ED_{50} = 3.3 \times 10^{-7}$ M), dose dependent, and stereospecific. Because diabetes represents a catabolic, lipolytic state with numerous vascular complications, we examined the action and regulation of monobutyrin in insulin-deficient diabetic rats. Serum levels of monobutyrin in streptozotocin-induced diabetic rats were greatly elevated compared to normal animals. At the same time, the retinal vessels of the diabetic animals develop a resistance to the vasodilatory activity of monobutyrin. These results demonstrate a role for monobutyrin in the control of vascular tone and suggest a possible involvement in the pathology of diabetes. (*J. Clin. Invest.* 1993. 92:2872–2876.) Key words: adipocyte • angiogenesis • diabetes • lipid • vasodilation

Introduction

Adipose tissue development and function depend intimately upon blood vessel formation and blood flow rates. 3T3-F442A cells have been shown to secrete angiogenic factors when they undergo adipocyte differentiation (1–3). One such factor, 1-butyryl-glycerol (monobutyrin), was originally purified from adipocyte-conditioned media based upon its ability to induce neovascularization in the chick chorioallantoic membrane assay (4). Subsequent studies have shown that the synthesis of this lipid is elevated during lipolysis (5) and that the pathway for monobutyrin production is tightly linked with the lipolytic pathway of triacylglycerol hydrolysis (6). This correlation, along with the in vivo observation that activation of lipolysis leads to increased blood flow in adipose tissue (7), suggested that monobutyrin may affect vascular tone.

We chose to use a video fluorescein angiogram assay to study the effect of monobutyrin on retinal circulation. The retina is a highly vascularized tissue whose hemodynamic changes can be directly monitored by angiography. By using sodium fluorescein dye as the indicator and the dye dilution method (8), one can observe noninvasively the in vivo effect of test substances on the retinal circulation with high resolution (9).

Our data indicate that monobutyrin treatment increases the retinal blood flow and causes vasodilation. In addition, insulin-dependent diabetic rats that undergo persistent lipolysis have elevated monobutyrin serum level and their retinal blood vessels develop a resistance to monobutyrin's vasodilatory effects.

Methods

Animals. Fluorescein angiography and monobutyrin serum level assays were performed using adult male albino Sprague-Dawley rats (Taconic Farms, Inc. Germantown, NY) weighing 200–250 g. Brown Norway and Long Evans rats were also used for fluorescein angiography. To induce insulin-dependent diabetes, animals were fasted for 12 h and 65 mg/kg of streptozotocin was injected intraperitoneally. The blood glucose level was between 300 and 400 mg/dl 1 wk after injection. To some animals, insulin treatment was given 1 wk after streptozotocin injection. Insulin (100 U regular insulin buffered with 7 mg/ml glutamic acid) was delivered intraperitoneally using Alzet mini-osmotic pump model 2001 (Alza Corp., Palo Alto, CA) at 1.0 μl/h for 7 d. All animals were handled and cared for according to Association for Research in Vision and Ophthalmology regulations regarding the use of animal models in research.

Video fluorescein angiography and blood flow measurements. The instrumentation and procedures were as described by Bursell et al. (10). Briefly, video fluorescein angiograms were recorded before and after infusion. Intravitreal infusion was performed by inserting a 10-μl Hamilton syringe needle into the vitreous. The insertion site was 1 mm posterior to the limbus. Insertion and infusion was performed under direct visualization and care was taken not to injure the lens or retina. The head of the needle was positioned directly over the optic disc region. At 10 min after infusion, 5 μl of fluorescein dye was injected into the jugular vein catheter while the time was marked. The recorded fluorescein angiograms were digitized on a frame-by-frame basis and analyzed to determine retinal vessel diameters and average circulation times.

All vessel measurements were made at a fixed radius from the center of the optic disc. At the vessel sites, the average vessel fluorescence was measured on a frame by frame basis to generate the dye dilution curves. The resulting artery and vein fluorescence data were then fit to a log normal distribution function (8). The values of the fitted parameters were used to determine mean arterial and venous circulation times. The difference between the mean venous and the mean arterial time was defined as the retinal mean circulation time (MCT).[1] Vessel diameters were measured before fluorescein dye injection in picture element units (pixels) using a zero crossing algorithm. Retinal blood flow is proportional to the sum of the squares of the arterial and venous diameters divided by the MCT. The units of blood flow in this case were in square pixels per second.

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1. *Abbreviations used in this paper:* AT, arterial appearance time; MCT, mean circulation time.

The arterial appearance time (AT) was defined as the time between dye injection and the first detectable appearance (vessel fluorescence intensity greater than the background intensity level by two times the standard deviation of the average background intensity) of the dye in the central retinal artery at the optic disc. The AT was used to assess any changes in systemic circulation.

Synthesis of stereospecific monobutyrin. The isomers were synthesized using the stereospecific starting compound *sn*-1 or *sn*-3 isopropylidene glycerol and butyryl chloride (Aldrich Chemical Co., Milwaukee, WI) essentially as described (11). After the reaction, the mixture was washed twice with 1 vol of a dilute HCl solution and neutralized by washing three times with 1 vol each of a saturated $NaHCO_3$ solution. The solution was then dried under N_2 gas and resuspended in methanol, and the isopropylidene blocking group was removed by incubation with Dowex cation exchange resin (Bio-Rad Laboratories, Richmond, CA) for 3 h. The resulting solution was dried under N_2 gas, resuspended in chloroform, and applied to a silica gel column (Biosil, Bio-Rad Laboratories). The column was washed with chloroform and monobutyrin was eluted by chloroform/methanol (9:1). This solution was dried under N_2 gas, resuspended in ether and monobutyrin allowed to crystallize at −70°C. The final product's identity and purity were confirmed by thin-layer chromatography with authentic monobutyrin, high-field proton-nuclear magnetic resonance, and mass spectroscopy (not shown). In addition, two separate preparations of the stereoisomers were examined in the retinal assay, yielding identical results.

Assay of monobutyrin serum level. Serum samples (1 ml) from normal or diabetic rats were extracted using 0.25 ml of chloroform/methanol (7:1) and centrifuged at 10,000 rpm for 10 min. Approximately 95% of monobutyrin remained in the aqueous phase. The aqueous phase was vacuum dried for 15 min at 37°C to remove the remaining chloroform. Monobutyrin was then extracted and quantitated as described by Wilkison et al. (5). Briefly, monobutyrin was partially purified using Sep-Pak C18 cartridges and converted to 1-butyryl-3-phosphoglycerol using [γ-^{32}P]ATP (New England Nuclear, Boston, MA) and glycerol kinase (Sigma Chemical Co., St. Louis, MO). The resulting radioactive phosphomonobutyrin was separated from other substances by thin layer chromatography and quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Results

Monobutyrin induces vasodilation in a dose-responsive manner. To determine whether or not monobutyrin affects blood flow, we employed an assay which utilizes video fluorescein angiography to evaluate retinal circulatory parameters. The angiograms were recorded so that the blood vessel diameters and circulation time could be accurately determined. The average time taken by the fluorescein dye to flow from the retinal arteries to veins is defined as MCT, which is inversely proportional to blood flow rate. As shown in Table I, monobutyrin appeared to be an efficient inducer of an increase in blood flow as MCT decreased by 0.43±0.23 s after monobutyrin injection. Using the MCT and the blood vessel diameters to determine the retinal blood flow rate revealed that monobutyrin caused a more than twofold increase in flow rate. This compound was more effective in this assay than 10^{-3} M histamine (0.34±0.29 s), a potent vasodilator (12). Glycerol, butyrate, a mixture of glycerol and butyrate (10^{-3} M each, not shown), or vehicle alone was unable to alter blood flow (Table I), showing that monobutyrin's biological activity was not due to simple osmotic or ionic effects. No change in systemic blood flow was observed as indicated by ATs (not shown). This effect was also observed in two other strains of rat (Long Evans and Brown Norway) with the same quantitative results, indicating monobutyrin-mediated vasodilation is not a strain-specific phenomenon.

The response to monobutyrin was also dose dependent with an EC_{50} of 4×10^{-6} M infused concentration (Fig. 1). While it is difficult to accurately determine the true concentration of monobutyrin in the vitreous, if one assumes total diffusion in the eye after injection (with a vitreal volume of 0.12 ml), the estimated concentration resulting from a 10-μl amount of a 4×10^{-6} M solution would be 3.3×10^{-7} M.

Only one enantiomer of monobutyrin causes vasodilation. Monobutyrin is a chiral molecule, and it is therefore of considerable interest to determine if monobutyrin's effects are stereospecific. Stereospecificity would imply specific ligand–protein interactions. To test this, we synthesized the enantiomers of monobutyrin and assayed their effects in the retinal assay. The *sn*-3-butyryl-glycerol gave an average decrease in the MCT of 0.5±0.25 s ($P = 0.0001$; $n = 13$ animals) which corresponds to a 27.8% decrease in MCT. This effect is comparable to that seen with the racemic mixture (see Table I). The other enantiomer, *sn*-1-butyryl-glycerol, failed to produce a statistically significant change in the retinal MCT. Of 12 animals assayed

Table I. Response to Various Compounds in Retinal Blood Flow Assay

	Monobutyrin (10^{-4} M)	Histamine (10^{-3} M)	Vehicle (PBS)	Glycerol (10^{-3} M)	Butyrate (10^{-3} M)
n	8	8	4	3	3
MCT (*s*)					
Preinfusion	0.90±0.10	0.87±0.23	0.92±0.1	0.85±0.10	1.10±0.10
Postinfusion	0.47±0.14	0.53±0.16	0.94±0.10	0.96±0.12	1.1±0.08
δ time (*s*)	−0.43±0.23	−0.34±0.29	−0.03±0.05	0.1±0.09	0.0±0.1
P value	0.001	0.015	0.4	0.24	1.0
Blood flow ($pixel^2/s$)					
Preinfusion	142.4±24.8	152.5±40.7	154.6±15.6	151.5±14.5	126.2±17.1
Postinfusion	340.4±83.6	317.6±126.8	150.9±22.0	138.7±25.3	126.1±18.6

Retinal MCTs and blood flow rates were measured. All compounds were suspended in PBS and injected intravitreally at the concentrations indicated. Glycerol and sodium butyrate were purchased from Sigma Chemical Co. Histamine was purchased from Sigma Chemical Co. *P* value was determined by the Student *t* test. *n* indicates the number of animals examined for each compound. The δ time represents the average difference in MCTs before and after injection.

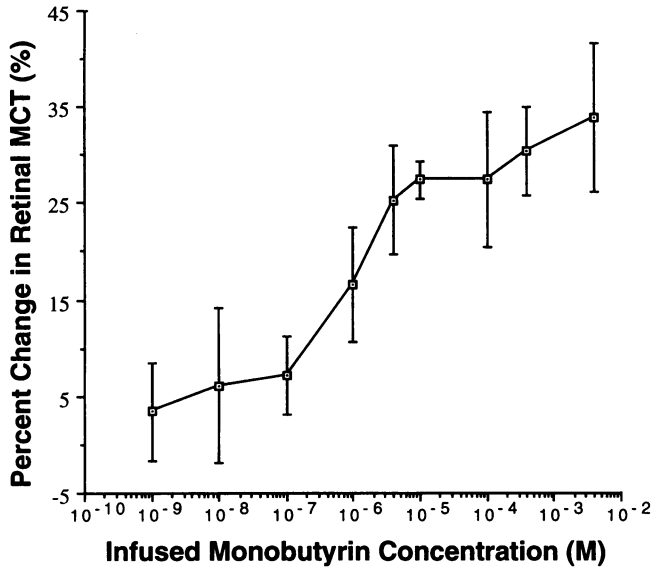

Figure 1. Monobutyrin dose-response curve. Different concentrations of monobutyrin was injected intravitreally and flow rates were measured after 10 min. At least three animals were assayed per monobutyrin concentration tested. Error bars indicate standard error.

with the *sn*-1-butyryl-glycerol enantiomer, only 5 demonstrated decreased MCTs. The average change in MCT of this group was 0.02±0.16 s.

The increased blood flow is primarily mediated by venule dilation. Changes in blood flow may be a result of the action of monobutyrin on arterial and/or venous systems. An advantage of the retinal assay system used here is the ability to determine if a vasoactive compound is acting upon venule or arterial vessels within the same organ. By examining where flow was most affected, we were able to show that monobutyrin exerted its effects primarily on venous vessels. The measurements were taken from a group of eight animals. The decrease in MCT was primarily due to a change in venous circulation time (0.35±0.19 s, $P = 0.0001$). The arterial circulation time was also decreased but not to the same extent (0.11±0.07 s, $P = 0.001$). Furthermore, direct measurements indicated that venous vessel diameters increased from 8.49±0.37 to 9.40±0.33 pixels in response to monobutyrin, representing a 10.8% increase. The arterial vessel diameters increased by a smaller degree from 7.33±0.25 to 7.81±0.38 pixels (a 6.5% increase). Both increases were statistically significant at the $P = 0.0001$ level using a paired t test to compare changes from preinfusion values.

Serum monobutyrin levels increase in diabetic rats. Diabetes is a metabolic disease accompanied by severe and often life-threatening disturbances in blood vessel biology (13, and see Discussion). The decreased insulin concentration in untreated insulin-dependent diabetes leads to a persistant lipolytic state. The interrelationship between monobutyrin synthesis and lipolysis suggests that monobutyrin serum levels may be elevated during such a pathophysiological state. To determine if monobutyrin is altered in this disease, the serum of normal and streptozotocin-induced diabetic rats was assayed for monobutyrin levels. Normal animals had a concentration of $8.71 \pm 0.35 \times 10^{-7}$ M ($n = 10$). However, assays of serum monobutyrin levels 1 wk after streptozotocin treatment showed an approximate threefold elevation ($2.9 \pm 1.06 \times 10^{-6}$ M, $P = 0.0001$; Table II) compared to the nondiabetic rats.

Diabetic rats fail to respond to monobutyrin. The elevation of monobutyrin serum levels in diabetic animals led us to examine whether or not there were alterations in the effect of monobutyrin on their retinal vessels. Streptozotocin-induced diabetic rats have a significantly prolonged MCT in comparison to normal rats (1.83±0.4 vs. 1.09±0.27 s for a normal animal), which is thought to be indicative of microvessel damage associated with the hyperglycemic state (10). When 10^{-4} M monobutyrin was tested, blood flow in the retinal vessels from 1-wk diabetic rats showed a complete resistance to monobutyrin-induced vasodilation (preinfusion MCT = 1.73±0.29 s and postinfusion MCT = 1.83±0.33 s, $P = 0.54$, Table II). Thus, insulin-deficient diabetic animals have alterations in monobutyrin levels and appear to have lost the ability to respond to this compound.

Insulin-treated diabetic rats have normal response to monobutyrin. The failure of diabetic rats to respond to monobutyrin could be caused by various reasons. To rule out the possibility that blood flow changes observed were due to direct streptozotocin toxicity, we treated the diabetic rats with insulin to determine if the resistance to monobutyrin was reversible.

As shown in Table III, after 1 wk of insulin treatment, the base line blood flow rate was partially recovered (comparing 119.75±51.83 to 142±24.8 pixel^2/s of normal rats). The response to monobutyrin infusion, however, was normal both in the decrease of MCT and the approximately twofold increase of blood flow rate. In comparison, 2-wk diabetic rats had reduced base line blood flow rate and no statistically significant response to monobutyrin infusion was detected. Serum assays also showed that blood monobutyrin concentrations decreased to that of normal rats after insulin treatment (data not shown). The effect of monobutyrin on rats with a longer duration of diabetes and treatments with various insulin therapy is currently under investigation.

Discussion

We report here that monobutyrin is a potent vasodilatory agent whose production directly responds to the lipolytic state of adipose tissue. Not only are monobutyrin levels elevated in the early stages of diabetes in rats, but these animals also show an altered response to monobutyrin in retinal vessels. Since adipo-

Table II. Serum Monobutyrin Levels and Response to Monobutyrin in Diabetic Rats

	Serum monobutyrin concentration	MCT decrease (10^{-4} M monobutyrin)
	M	*S*
Normal	$8.71 \pm 0.35 \times 10^{-7}$ ($n = 10$)	0.43±0.23 ($n = 8$, $P = 0.001$)
Diabetic	$2.90 \pm 1.06 \times 10^{-6}$ ($n = 10$)	−0.1±0.33 ($n = 7$, $P = 0.54$)

Streptozotocin-induced diabetic rats and age paired normal rats were assayed for serum monobutyrin levels. Similarly, diabetic and age-paired normal rats were assayed for vasodilatory response mediated by monobutyrin. P value was determined by the Student t test. n, number of animals examined for each experiment.

Table III. Insulin Treatment on Diabetic Rats Recovered the Monobutyrin Response on Retinal Blood Flow

	Insulin-treated diabetic rats (n = 4)	Untreated diabetic rats (n = 7)
Blood glucose (*mg/dl*)	80.5±19.84	431.14±24.75
MCT (*s*)		
Preinfusion	0.97±0.72	1.40±0.68
Postinfusion	0.48±0.13	1.41±1.12
δ time (*s*)	0.49±0.63	−0.01±0.99
P value	0.02	0.27
Blood flow (*pixel²/s*)		
Preinfusion	119.75±51.83	89.85±58.84
Postinfusion	213.22±37.84	112.29±67.72

Streptozotocin-induced 1-wk diabetic rats were treated with insulin for 7 d as described in Methods. Age-paired 2-wk diabetic rats were used as the untreated diabetic control group. Retinal MCTs and blood flow rates were measured before and after monobutyrin (10^{-4} M in PBS) infusion. n, number of animals examined. P value was determined by the Student t test.

cytes are an important regulator of energy balance and one of the major target cells for insulin, the connection between fat cells and the development of diabetic vascular pathology is extremely important. Given these correlations, it is reasonable to suggest that the elevated serum concentrations of monobutyrin may be a contributory element in some of the vascular abnormalities observed in diabetic animals.

Diabetes mellitus is a chronic metabolic disease associated with microvascular complications, such as nephropathy and retinopathy. Diabetic nephropathy is associated with both glomerular expansion and renal hypertension (14). Diabetic retinopathy involves retinal microaneurysms, increased capillary proliferation, and abnormal vessel structures leading to retinal detachment and blindness (15). Although the vascular pathology may be caused by multiple factors, small vessel hemodynamic changes are observed before any morphological changes (16). In the early stage of diabetes, reduction in blood flow was shown in diabetic patients with no retinopathy (17, 18). Similarly, reduced blood flow was found in 1-wk diabetic rats and dogs 5 mo after the onset of diabetes, which was long before signs of diabetic retinopathy occurred (10, 19). Subsequently, increased retinal blood flow was observed as background diabetic retinopathy proceeded in humans (20). Additionally, vessels from diabetic patients and animal models have altered responses to vasoactive agents (21, 22). Thus, these findings suggest that functional damage relating to blood flow exists in microvessels early in the development of diabetic vascular diseases. However, the direct cause of these vessel changes is not clear.

The result that the vasodilatory effect is specifically mediated by the *sn*-3-butyryl-glycerol isomer suggests that monobutyrin interacts with a receptor or enzyme system. The increased monobutyrin levels in diabetic rats may down-regulate or desensitize a specific receptor/protein which mediates monobutyrin's effects, in a manner similar to that seen with the adrenergic receptors (23, 24). The desensitization could account for the ineffectiveness of monobutyrin to cause retinal vasodilation. Down-regulation of the putative receptor of monobutyrin in retinal vessel may also account for the decrease in blood flow seen in 1-wk diabetic rats (10), assuming this putative receptor for monobutyrin is essential for vascular tone. The recovery of the monobutyrin response after insulin treatment indicates that the initial failure is not due to streptozotocin toxicity and the resistance to monobutyrin is reversible at early stage of diabetes. Whether the loss of effect on diabetic rats is the result of persistent high serum monobutyrin level or vascular damages caused by other metabolites associated with hyperglycemia is not clear based on our current data.

Interestingly, the increased severity of diabetic retinopathy is correlated with increasing venous abnormalities in humans (25), consistent with monobutyrin's preference for venal dilation (see Results). In addition, monobutyrin is also angiogenic (4) and therefore may play a role in the damaging vasoproliferation that occurs in the advanced stages of diabetic retinopathy. Preliminary studies suggest that high serum monobutyrin concentrations are associated with progressive proliferative retinopathy in humans (Halvorsen and Spiegelman, unpublished observations). In any case, it remains clear that monobutyrin does have potent vasodilatory effects and the role of this novel lipid in normal physiology and diabetic pathophysiology needs further examination.

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